Measurement and Clinical Applications of Homocysteine and Methylated Arginines

A Thesis submitted by

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This work is dedicated to

my Mother, my Brother and my Grandmother,

with love

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I would also like to thank my dance teacher, Caroline, for keeping my spirits lifted whilst carrying out this work.

DECLARATION

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

G R Weaving

ABBREVIATIONS

ADMA	asymmetric dimethylarginine
ANOVA	analysis of variance
CF-PWV	carotid femoral pulse wave velocity
CHD	coronary heart disease
CVD	cardiovascular disease
DDAH	dimethylarginine dimethylaminohydrolase
GFR	glomerular filtration rate
hcy	homocysteine
HPLC	high performance liquid chromatography
MDRD	modification of diet in renal disease
MMA	monomethylarginine
MS	mass spectrometry
OPA	o-phthaldialdehyde
SDMA	symmetric dimethylarginine
thcy	total homocysteine

AIMS

1) to develop a tandem mass spectrometric method for the simultaneous measurement of homocysteine, cysteine and methionine in human plasma and urine, suitable both for research into their interrelationships and also for application in the routine clinical laboratory

2) to develop a tandem mass spectrometric method for the simultaneous measurement of ADMA, SDMA, MMA and arginine in human plasma and urine, suitable both for research into their interrelationships and also for application in the routine clinical laboratory

3) to examine pre-analytical variables (eg sample type, patient demographics) which may determine the reliability of the above analyses and/or the interpretation of the results obtained

4) to test the hypothesis that an increase in plasma homocysteine leads to an increase in plasma ADMA

ABSTRACT

Homocysteine is an amino acid formed by the metabolism of methionine. Increased plasma homocysteine concentrations are associated with cardiovascular disease, and it has been suggested that homocysteine lowering therapy may reduce cardiovascular risk. Plasma homocysteine measurements are frequently requested by clinicians investigating patients with vascular disease. A mechanism for homocysteine causing vascular disease has not yet been proven, but one possibility is that an elevated plasma homocysteine concentration may lead to the accumulation of asymmetric dimethylarginine (ADMA), a naturally occurring amino acid that inhibits nitric oxide synthase, resulting in impaired nitric oxide production, and therefore vascular dysfuntion.

The aim of this project was to develop analytical methods suitable for the measurement of homocysteine and related metabolites in a routine clinical laboratory, and two methods have been established; i) for homocysteine, cysteine and methionine and ii) for asymmetric dimethylarginine, symmetric dimethylarginine (SDMA, a sterioisomer of ADMA), monomethylarginine (MMA) and arginine. A novel feature of the method for ADMA is that the use of unique daughter ions allows the determination of both ADMA and SDMA without the need to separate the isomers chromatographically. In addition, the synthesis and application of isotopically labelled SDMA, for use as an internal standard, is described for the first time.

When the methods were applied to the analysis of routine clinical samples no association was detected between plasma total homocysteine and plasma ADMA concentrations. Measurements were also performed on samples from patients enrolled in a clinical trial investigating the progression of vascular dysfuntion, as measured by carotid-femoral pulse wave velocity (CF-PWV), in chronic kidney disease. Again no association could be found between plasma total homocysteine and plasma ADMA concentrations. In addition plasma total homocysteine was not a determinant of CF-PWV.

These findings do not support the hypothesis that hyperhomocysteinaemia causes vascular disease by increasing ADMA concentrations.

1. Introduction

1.1. Cardiovascular disease

1.1.1. Cardiovascular disease and mortality

Diseases of the heart and circulatory system (cardiovascular disease, CVD) are a major health care issue, accounting for approximately 35% of deaths in the United Kingdom in 2006[1] (figure1.1). CVD is one of the major forms of premature death (death before age 75); in 2006 22% of premature deaths in women and 30% in men were due to CVD. The cost to the United Kingdom health care system in 2006, due to CVD, was approximately £14.4 billion.

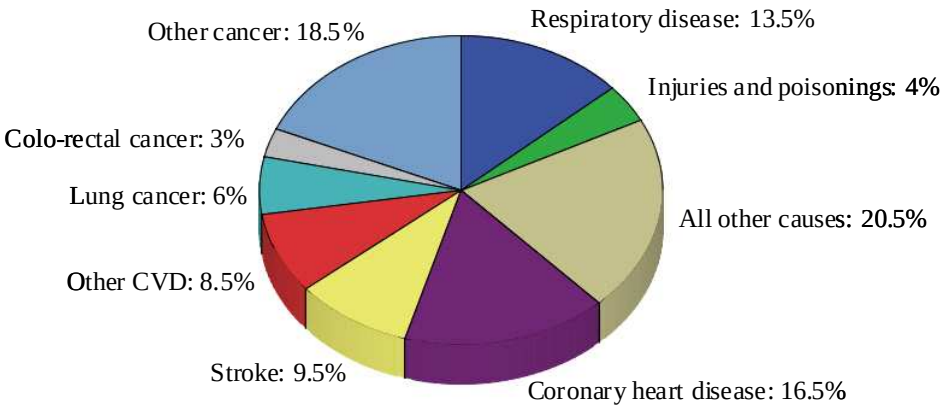


Figure 1.1. Causes of mortality in the United Kingdom, 2006. The figures are for men and women combined, using data taken from CHD UK Mortality. British Heart Foundation Statistics Database[1].

1.1.2. The Pathogenesis of Atherosclerosis

Atherosclerosis is now regarded as an inflammatory disease, the mechanisms of which have been reviewed[2]. In the response-to-injury hypothesis of atherosclerosis endothelial dysfunction is caused by damage to the vasculature, causes of which may include hypertension, damage by free radicals or oxidised LDL, diabetes mellitus, or infections (eg herpes virus). The resulting endothelial dysfunction leads to compensatory responses that alter the normal haemostatic properties of the endothelium, with changes in its permeability and increased adhesiveness for leukocytes and platelets[2]. The endothelium also develops procoagulant instead of anticoagulant properties with the formation and release of cytokines and growth factors[2]. If the initial injury is not repaired the inflammatory response can continue with a subsequent migration of smooth-muscle cells into the site of inflammation and an intermediate lesion forms, macrophages and lymphocytes then begin to emigrate from the blood into the lesion with further release of cytokines and growth factors[2]. As the lesion grows restructuring occurs so that it becomes covered by a fibrous cap covering a core of lipid and necrotic tissue, intruding into the vessel lumen and altering the flow of blood; thinning of the fibrous cap can lead to rupture of the lesion and thrombosis[2].

Traditional risk factors for the development of atherosclerosis include age, gender, hypertension, dyslipidaemia, hyperglycaemia and smoking, however there is a large proportion of CVD that can not be explained by these risk factors alone[3], in particular they do not adequately account for the substantial increase in cardiovascular events and mortality associated with chronic kidney disease[4]. Considerable research has therefore focused on identifying and evaluating novel cardiovascular risk factors[5] (table 1.1).

Emerging risk factors for cardiovascular disease.	
C-reactive protein	Interleukins
Serum amyloid A	Vascular and cellular adhesion molecules
Soluble CD40 ligand	Leukocyte count
Fibrinogen	Plasminogen activator inhibitor 1
D-Dimer	Tissue plasminogen activator
Factors V, VII, VIII	Small dense LDL
LDL and HDL subtypes	Apolipoproteins A1 and B
Homocysteine	Lipoprotein associated phospholipase A_2
Microalbuminuria	Creatinine (glomerular filtration rate)
Cystatin C	Infectious agents
ApoE genotype	Fibrinopeptide A
Remnant lipoproteins	von Willebrand factor antigen

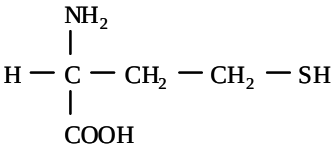
Table 1.1. Emerging risk factors for cardiovascular disease. Adapted from Myers et al[5].

1.2. Homocysteine

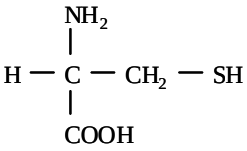
1.2.1. The Biochemistry of Homocysteine

Homocysteine is a thiol containing amino acid which exists as two enantiomers (D- & L-). Amino acid metabolism in humans is restricted to L- isomers and D- isomers are not metabolised: throughout this thesis the L-isomer is implied for all amino acids. Homocysteine is not obtained from the diet but is derived from the metabolism of methionine. Homocysteine lies at a metabolic branch-point: it may be further metabolised to cystathionine and then cysteine (transsulphuration) which requires vitamin B6 as a cofactor, or be remethylated to methionine, either by the vitamin B12 dependent enzyme methionine synthase, or by betaine-homocysteine methyltransferase. The structures of homocysteine, cysteine and methionine are shown in figure 1.2. and the formation and metabolism of homocysteine in figure 1.3.

homocysteine



cysteine



methionine

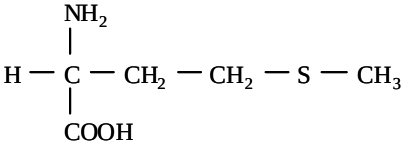


Figure 1.2. Structures of homocysteine, cysteine and methionine.

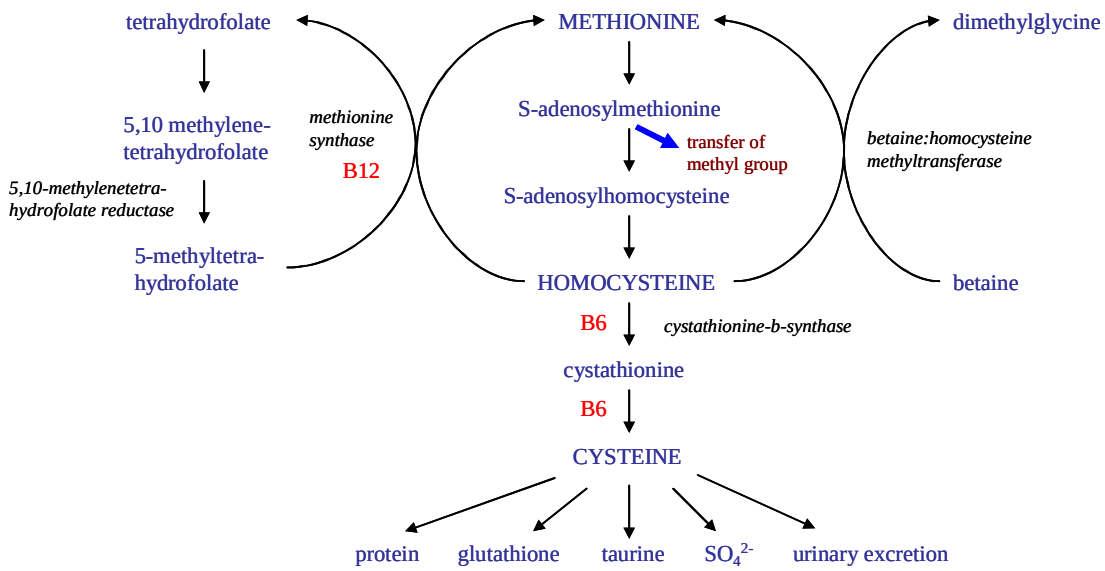


Figure 1.3. The metabolism of homocysteine. Homocysteine is formed by the transfer of a methyl group from methionine. It may further metabolised to cysteine in vitamin B6 dependent reactions (transsulphuration) or reconverted to methionine (remethylation) by a) *betaine:homocysteine methyl transferase* or b) the vitamin B12 dependant enzyme *methionine synthase*.

In plasma cysteine and homocysteine exist in a variety of forms, as reduced thiols, as disulphides, and as mixed disulphides. They are also found bound to protein. Approximately 4% of cysteine is in the reduced form, while 32% exists as a variety of disulphides and the remaining 64% is protein bound. In the case of homocysteine approximately 2% is reduced, 16% is in the form of disulphides and 82% is protein bound[6]. The predominant disulphides are cystine (formed from two cysteines), homocystine (formed from two homocysteines) and cysteine-homocysteine mixed disulphide.

1.2.2. Classic Homocystinuria

Homocystinuria was first described in 1963 in two sisters, aged 4 and 6, who presented with severe mental retardation and a history of seizures[7]. Both children had fine, sparse blonde hair and blue eyes, did not resemble other members of their family, and examination

of both children's eyes showed that the lenses were “dislocated backwards and slightly upwards”[7] Homocystine and methionine were shown to be increased in plasma, with a low-normal cystine concentration, and these finding, together with the appearance of the children, led the authors to initially propose that "the pathogenesis of the disease was due to a cystine deficiency resulting from a metabolic block in the breakdown of methionine and cystine"[7]. At presentation the younger sibling was on the 3rd percentile for height and 25th percentile for weight, and treatment with oral cystine supplementation was initiated to see if it would induce a growth spurt and encourage improvement in hair growth, but with no effect. The lack of response following cystine treatment combined with further studies involving the measurement of urine homocystine and methionine following a methionine load led to the supposition that the disease was most likely be a result of defective cellular transport of methionine.

In 1964 Mudd et al demonstrated reduced activity of cystathionine synthase activity in liver tissue obtained at biopsy of an 8 year old homocystinuric female[8] and suggested this enzymic block could explain increased plasma concentrations of both homocystine and methionine.

Post-mortem examinations of a male child who died at 7½ weeks revealed increased plasma and urine homocystine with low concentrations of methionine and normal tissue activity of cystathionine synthase and betaine-homocysteine methyltransferase, but reduced activity of methionine synthase[9]. At autopsy extensive microscopic lesions were observed throughout the arterial tree[10], bearing a close similarity to those that had been noted in homocystinuria due to cystathionine synthase deficiency. This similarity, together with the only common biochemical finding of increased homocystine concentrations, led McCully to propose that increased plasma homocystine is directly responsible for the development of vascular disease[10].

In 1985 a questionnaire-based survey of physicians involved in the identification and care of 629 patients with homocystinuria, 472 of whom were identified on the basis of clinical features, the remaining being identified through screening programmes or investigated due

to having an affected sibling, showed that the age of onset of disease and the severity of clinical manifestations varied widely amongst affected individuals[11]. Of the individuals who had been identified clinically, common features were ectopia lentis (86%), mental retardation (56%) Marfanoid characteristics (37%) and early thromboembolic disorders (16%). The age at which therapy commenced varied (with age at presentation) and in those individuals where treatment was not initiated early the chance of a thromboembolic event was 25% by age 16 and 50% by age 29. Patients were classified as to their response to vitamin B6 therapy (ie by a lowering of plasma homocysteine): 36.7% being responsive, 36.7% being non-responsive, 10.7% showing an intermediate response and the remaining 15.9% not being classified due to insufficient information. Treatment by vitamin B6, in responsive individuals, resulted in a statistically significant reduction in the number of thromboembolic events but the authors stated that given the relatively small number of events that had been observed this finding should be considered suggestive rather than definitive[11].

A similar multicentre study carried out in 2001 examined data concerning 158 homocystinuric patients who had been treated and followed for a mean of 17.9 years (resulting in 2822 patient years)[12]. Where patients had shown a lack of response to vitamin B6 therapy (a positive response being defined as lowering plasma homocysteine to less than 20 μmol/L) dietary restriction of methionine and/or the use of betaine had been employed. Based on the findings of the 1985 survey 112 vascular events were expected had the patients not been treated; a total of 17 occurred in 12 patients. The difference between predicted and observed events was statistically highly significant ($p<0.001$) and the study concluded that "long-term treatment to lower the markedly elevated plasma levels of homocysteine seen in homocystinuria due to cystathionine-β-synthase deficiency is effective in reducing the potentially life-threatening vascular risk".

The frequent occurrence of severe thromboembolic disease at an early age in homocystinuria, and the findings that this seems to be lessened by the reduction of plasma homocysteine by vitamin treatment has led to the 'homocysteine theory of atherogenesis': homocysteine is in itself atherogenic and increased plasma concentrations may represent a risk factor for vascular disease in the general population.

1.2.3. Hyperhomocysteinaemia in the General Population

Homocystinuria has an estimated worldwide incidence of 1 in 335,000[13]. The incidence varies with the population studied, ranging from 1 in 65,000 in Ireland, where a national screening programme has been in place since the late 1960s, to 1 in 900,000 in Japan[13]. Although homocystinuria is a rare disorder there is a large number of other causes of mild to severe hyperhomocysteinaemia, including polymorphisms in genes encoding for methylenetetrahydrofolate reductase (MTHFR)[14], which forms part of the homocysteine remethylation cycle, various diseases and lifestyle factors[15,16] and also a number of common therapeutic drugs[17] (table 1.2).

Determinant	Effect
Inherited	
homozygosity for CBS defects	++++
heterozygosity for CBS defects	++
homozygosity for MTHFR defects	++++
heterozygosity for MTHFR defects	++
Clinical conditions	
folate deficiency	+++
vitamin B12 deficiency	++++
vitamin B6 deficiency	++
renal failure	+++
hyperproliferative disorders	++
hypothyroidism	++
Lifestyle factors	
smoking	+
coffee consumption	+
alcohol consumption, moderate/excessive	-/+
Drugs	
antiepileptics	++
cholestyramine	++
fibrates	++
L-dopa	++
metformin	++
methotrexate	++
nicotinic acid	++
nitrous oxide	+++

Table 1.2. Determinants of hyperhomocysteinaemia. + is an increase in plasma homocysteine within the reference range (5-15 μmol/L), ++ is mild to moderate hyperhomocysteinaemia (15-30 μmol/L), +++ is intermediate hyperhomocysteinaemia (30-100 μmol/L) and ++++ is severe hyperhomocysteinaemia (>100 μmol/L)[15,16,17].

1.2.4. Epidemiological Evidence for Homocysteine as a Vascular Risk Factor

1.2.4.1. *Case Controlled and Prospective Studies*

The results of 27 studies examining the relationship between vascular disease and homocysteine were subject to meta-analysis in 1995 by Boushey et al[18]. The studies had been carried out at various times during the period 1974-1994. Three were prospective studies, five cross-sectional, and nineteen case-controlled studies and had examined cardiovascular disease, cerebrovascular disease, peripheral vascular or a combination of these. The studies varied considerably in the number of cases and controls, age of subjects, preparation of subjects (fasting, non-fasting, post-methionine load) and analyte measured (homocystine, cysteine-homocysteine mixed disulphide, total homocysteine), but all were considered to have provided good quality data[18]. Fourteen out of seventeen studies supported homocysteine as being a risk factor for cardiovascular disease with an overall odds ratio of 1.7 (95% confidence interval 1.5 - 1.9). Nine out of eleven studies showed an association between homocysteine and cerebrovascular disease, overall odds ratio 2.5 (95% confidence interval 2.0 - 3.0) and all nine studies investigating peripheral vascular disease showed a positive association with elevated plasma homocysteine concentrations, overall odds ratio 6.8 (95% confidence interval 2.9 - 15.8).

The largest case controlled study performed so far is that of the European Concerted Action Project (reported in 1997), a multicentre study carried out in a total of 19 centres in 9 European countries[19]. This included a total of 750 cases of vascular disease (cardiac, cerebral and vascular) and 800 controls. All samples were taken from subjects in the fasting state and also 6 hrs post methionine load (100 mg/kg body weight) and were all analysed at only one centre. Positive associations were found between plasma homocysteine and all three forms of vascular disease investigated, with the relative risk for vascular disease being given as 2.2 (95% confidence interval 1.6 - 2.9). The risk conferred by homocysteine was independent of other risk factors, but interactions between smoking and also hypertension were noted, with a multiplicative effect upon those risks.

Two further meta-analyses, both published in 2002, were both less supportive of there being an association between plasma homocysteine and vascular disease[20,21], one concluding that "elevated homocysteine is at most a modest independent predictor of ischaemic heart disease and stroke risk in healthy populations"[21]. Pertinent details of these two studies are summarised in table 1.3. The study of Ford et al noted that case controlled studies provided a stronger degree of association between plasma homocysteine and vascular disease as compared with cohort and nested cohort studies. The authors could give no explanation for this observation but did note a number of difficulties in combining the data from individual reports. Results had been given in various ways, different reference ranges and cut-off values had been used, the boundary values used for data analysis (eg quartiles) were not always stated, odds ratios had not always been calculated in the original papers and had to be calculated by the reviewing authors. In addition, details about sample collection procedures were often lacking as was information regarding the methods used for homocysteine measurement and control procedures to ensure accuracy of the analysis. An examination of the analytical performance of laboratories participating in a quality assurance programme in 1999[22] (comparable to the date at which the meta-analyses were published) concluded that only 9 of the 34 participating laboratories achieved the minimum imprecision goal, the between laboratory imprecision for HPLC analysis was 8.7% and that there was a need for improvement in the imprecision of analysis. The lack of precision demonstrated by this scheme could represent a major confounder in meta-analyses.

In the meta-analysis performed by the Homocysteine Studies Collaboration (HSC) the difficulties in amalgamating data had been circumvented by asking the original investigators to provide information and data on each participant but no consideration was given as to the possible effects that might arise through the use of different methods of homocysteine measurement. Although this study concluded that an increased plasma homocysteine was at best a modest risk factor for vascular disease it also stated that "the implications for public health of decreasing the population mean levels of homocysteine could still be substantial"[21].

In both meta-analyses certain groups of patients had been excluded, in particular those with diabetes, renal impairment, and systemic lupus erythematosus (SLE), in an effort to reduce confounding factors, but as these populations show a high incidence of vascular disease a particular focus on these groups may be warranted.

	Ford et al[20]	The Homocysteine Studies Collaboration[21]
Search period	1966 - 1999	1966 - 1999
Publications reviewed (n)	57	30
Study divisions	cohort (3) nested case controlled (12) case controlled (42)	retrospective (18) prospective (12)
Conditions examined	coronary heart disease and cerebrovascular disease	ischaemic heart disease and cerebrovascular disease
Information given regarding number of subjects	CHD 5518 cases 11068 controls CVD 1817 cases 4787 controls	Total population 16786 Ischaemic heart disease events 5073 Stroke events 1113
Homocysteine measurements	Studies either reported fasting, or post methionine load, homocysteine concentration	Studies reporting free homocysteine measurements, and those performed post methionine load, were excluded
Clinical criteria for excluding studies	Special populations excluded including patients with cardiovascular disease, diabetes, systemic lupus erythematosus and those receiving dialysis	Special populations excluded including patients with pre-existing cardiovascular disease, diabetes, renal impairment, systemic lupus erythematosus
Quality of analysis	Individual studies scored for quality and results inspected accordingly. Investigation of publication bias suggested that negative studies had not been selectively omitted	No report on quality of individual studies or investigation of possible selection bias
Results	Odds ratios reported as <u>increased risk</u> associated with a 5 μmol/L increment in plasma homocysteine For CHD: cohort 1.06 (0.99 - 1.13) nested cohort 1.23 (1.07 - 1.41) case controlled 1.70 (1.50 - 1.93) For CVD: cohort 1.10 (0.94 - 1.28) nested cohort 1.58 (1.35 - 1.85) case controlled 2.16 (1.65 - 2.82)	Reported as <u>reduced risk</u> for homocysteine concentrations in lowest quartile For IHD: 0.89 (0.83 - 0.96) For CVD: 0.81 (0.69 - 0.95) Stronger associations were observed in retrospective studies of homocysteine measured after onset of disease compared with prospective studies where individuals has no history of vascular disease when blood was collected
Conclusions	"prospective studies offer weaker support than case-control studies for an association between homocysteine and cardiovascular disease"	"elevated homocysteine is at most a modest independent predictor of IHD and stroke risk in healthy populations"

Table 1.3. Summary details of two meta-analyses investigating homocysteine as a vascular risk factor[20,21].

1.2.4.2. *Evidence From Studies on MTHFR Polymorphisms*

MTHFR catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which then acts as a substrate for methionine synthase in the remethylation of homocysteine. A number of single nucleotide polymorphisms of the genes encoding for MTHFR have been identified including a C to T change at position 677 (C667T) and an A to C change at position 1298 (A1298C)[23]. The C667T polymorphism is relatively common: 47% homozygous CC, 43% heterozygotes CT, 10% homozygous TT[24]. C667T polymorphisms have been shown to result in reduced enzyme activity[14] and increased plasma homocysteine[25]. The variant MTHFR resulting from the C667T polymorphism is often referred to as thermolabile MTHFR as a result of the way in which it was originally identified: variant MTHFR retaining 7 - 17% activity following heating at 46 ^{O}C for five minutes as compared with 'normal' MTHFR which retains 20 - 50% activity after heating[14].

A large number of studies have been carried out to investigate if there is an association between the presence of the C677T polymorphism (and therefore elevated plasma homocysteine) and vascular disease. In these genetic based studies it is assumed that the distribution of genes is effectively random and independent of other cardiovascular risk factors (smoking, increased blood pressure, increased lipids) that may be confounding factors in other types of study[29]. Individual studies have been subject to a number of meta-analyses[24, 25, 26, 27, 28, 29, 30], details of which are given in table 1.4. The majority of studies show a positive (though sometimes small) association between genotype and the form of vascular disease examined. One notable feature is the differences that occur when data is stratified according to geographical region, in particular North America consistently shows no association between genotype and vascular disease. It has been suggested that the differences may due to a publication bias[30], with some geographical regions tending to report only strongly positive studies. An alternative explanation may lie in the fact that individuals with the TT genotype only demonstrate hyperhomocysteinaemia when folate deficient. In a study performed by Jacques et al[31] subjects with the TT genotype and folate lower than the study median value had significantly higher plasma homocysteine

concentrations than subjects with similarly low folate but with the 'normal' CC genotype ($p<0.05$). There was no difference in plasma homocysteine between subjects with TT genotype and those with CC genotype when folate concentrations lay above the median value. In addition mandatory fortification of grain products with folic acid was instigated in North America, in order to reduce the incidence of neural tube defects, in 1996[32] and was essentially complete by 1997. Jacques et al[32] assessed the effects of this fortification by comparing results from samples taken before fortification, with those taken after, for 350 subjects enrolled in the Framingham Offspring Study cohort. Of the 350 subjects, 248 were not taking vitamin supplements, and for these the incidence of folate deficiency (defined as folate < 7 nmol/L) was decreased from 22.0 to 1.7% following fortification and the prevalence of high homocysteine (defined as > 13 μmol/L) was reduced from 18.7 to 9.8%. It may be that the lack of association between the C677T mutation and vascular disease in studies of North American populations would support the fact that the mutation only causes hyperhomocysteinaemia in the presence of folate deficiency and that this has largely been eliminated in this geographical region.

Reference	Number of studies	Disease investigated	Geographical Region	Number of cases/controls		Odds ratio	95% confidence interval
Studies showing a positive association with genotype for at least one group							
Wald et al (2002)[24]	72	IHD, DVT, stroke	not stratified	12193	11945	IHD 1.21	1.06 - 1.39
				3439	5063	DVT 1.29	1.08 - 1.54
				1217	not given	stroke 1.31*	0.80 - 2.15
Klerk et al (2002)[26]	40	CHD	Europe (22)	6207	8343	1.14	1.01 - 1.28
			N. America (10)	3146	2532	0.87*	0.73 - 1.05
			Overall	11162	12758	1.16	1.05 - 1.28
Den Heijer et al (2005)[27]	53	Venous thrombosis	Europe (30)	5300	7382	1.15	1.02 - 1.30
			N. America (11)	1738	2108	1.03*	0.82 - 1.29
			Other (12)	1326	2978	1.60	1.27 - 2.02
			Overall	8364	12468	1.20	1.08 - 1.32
Cronin et al (2005)[28]	32	ischaemic stroke	not stratified	6110	8760	1.37	1.15 - 1.64
Lewis et al (2005)[30]	80	CHD	Europe (41)	17275	21313	1.08*	0.99 - 1.18
			N. America (15)	3714	3969	0.93*	0.80 - 1.10
			Middle East (5)	971	1316	2.61	1.81 - 3.75
			Asia (16	2755	4735	1.23	0.94 - 1.62
			Australia (3)	1285	480	1.04*	0.73 - 1.49
			Overall	26000	31813	1.14	1.05 - 1.24
Studies showing no association with genotype							
Brattström et al (1998)[25]	23	CVD	not stratified	5869	6644	1.12	0.92 - 1.37
Kelly et al (2002)[29]	19	ischaemic stroke	not stratified	2788	3962	1.23	0.96 - 1.58

Table 1.4. Summary details of meta-analyses investigating MTHFR genotype as a vascular risk factor.

1.2.4.3. *Intervention Studies*

A number of intervention trials to assess whether homocysteine lowering therapy decreases the risk of vascular disease have now been reported[33, 34 ,35 ,36, 37, 38] and the salient features of these are given in table 2.4. All these studies concluded that treatment had no effect on vascular outcomes; these findings have generally met with disappointment and suggestions that increased plasma homocysteine is a marker of vascular disease and not causal[39]. There are, however, a number of criticisms in these studies both in terms of the conclusions drawn and study design.

For the VISP trial, after the initial findings had been published[33] the results were subject to further, sub-group analysis. After removal of subjects with impaired B12 absorption and/or significant renal impairment a 21% reduction in vascular events was noted in subjects receiving high-dose therapy as compared with those in the low-dose therapy group[40].

In the HOPE-2 trial a subgroup analysis showed that fewer patients receiving treatment had a stroke compared to those in the placebo group (relative risk, 0.75; 95% confidence interval, 0.59 - 0.97). It should be noted that in this trial not all participants had measurements of homocysteine and folate performed, but only a random selection: 60% at baseline, 21% at two years and 19% at five years (for both treated and placebo groups), ie at follow-up, data was incomplete for approximately 80% of participants.

Inclusion criteria for five of the six studies in table 1.5 was the existence of established vascular disease, and it is possible that homocysteine lowering therapy at this stage may not reverse any damage already caused to the vascular system. In view of this, whilst these trials may have shown there was no benefit in secondary prevention of vascular disease, care must be taken inferring that homocysteine lowering therapy would have no benefit in primary prevention.

Trial and period of study	No. of subjects	Inclusion criteria	Treatment groups	Effect on plasma homocysteine concentration (μmol/L)	Follow-up period	Results
VISP[33] 1996 -2003	3680	Nondisabling ischaemic stroke, age ≥ 35 years + plasma homocysteine in top quartile of North American population (threshold changed during the study)	High dose B6, B12 & folate vs low dose	2.3 decline from baseline of 13.4 in high-dose group	2 years	No effect on recurrent cerebral infarction, CHD or death.
HOPE-2[35] 2000 - 2005	5522	Age ≥ 55 years + vascular disease or diabetes along with other risk factors	Folate, B6, B12. Placebo	12.2 → 9.7 12.2 → 12.9	5 years	No effect on the combined endpoint of cardiovascular death, myocardial infarction and stroke
NORVIT[34] 1998 - 2004	3749	Acute myocardial infarction ≤ 7 days	Folate, B12, B6 Folate, B12 B6 Placebo	13.1 → 9.5 12.9 → 9.8 13.3 → 13.3 13.2 → 13.6	3.5 years	No effect on the combined endpoint of myocardial infarction, stroke and sudden death. Increased risk in the folate + B12 + B6 group
HOST[36] 2001 - 2006	2056	End stage renal disease or creatinine clearance ≤ 30 mL/min + homocysteine ≥ 15 μmol/L	Folate, B12, B6 Placebo	21.5 → 15.3 21.4 → 20.6	5 years	No effect on all cause mortality, MI or stroke.
WAFACS[38] 1998 - 2005	5442	Women > 40 years or post-menopausal, cardiovascular disease or three risk factors	Folate, B12, B6 Placebo	12.1 → 9.8 12.5 → 11.8	7.3 years	No effect on the combined endpoint of myocardial infarction, stroke, coronary revascularisation, cardiovascular death.
WENBIT[37] 1999 - 2006	3096	Coronary angiography for suspected coronary disease or aortic stenosis	Folate, B12, B6 Folate, B12 B6 Placebo	10.8 → 7.6 in first two groups with folate and B12 No change in B6 or placebo groups	38 months	No effect on composite endpoint of total mortality, nonfatal myocardial infarction, hospitalisation for unstable angina, or nonfatal thromboembolic stroke

Table 1.5. Major clinical trials investigating the effect of homocysteine lowering therapy on vascular outcomes. Data adapted from Joseph et al[41] in addition to individual references.

Four of the six studies were conducted (almost) exclusively in North America and Canada, where food fortification by folate is mandatory, and it could therefore be argued that the participants in the placebo groups were already receiving a degree of vitamin supplementation, and that the trials should be viewed in terms of *the potential benefits due to additional supplementation* rather than to supplementation per se.

The majority of participants in the trials were taking a variety of medications, with polypharmacy being common table 1.6. In some trials 12-29% of participants were also taking multivitamins in addition to those being administered as part of the trial. There were no differences with regard to medication or vitamin usage between the active groups and control groups in any of the trials. Given that many of the participants were being treated for vascular disease with these therapeutic agents the trials would largely only be able to detect the *additional* benefit of homocysteine lowering therapy. A more serious concern is that some of these agents could mask the (possible) toxic effects of homocysteine, or at least reduce the power of the studies to detect them. So far there is no proven mechanism for the (possible) atherogenic effects of homocysteine (which will be discussed in detail below) but it has been demonstrated that homocysteine a) has a procoagulant effect[42], which could possibly be reduced by aspirin therapy and b) reduces the production of nitric oxide, which could possibly be countered by statin therapy; many statins having been shown to increase nitric oxide production[43]. These issues were not addressed in the studies and caution should therefore be exercised in drawing conclusions beyond the lack of effect noted in the targeted population.

	VISP	HOPE-2	NORVIT	HOST	WAFACS	WENBIT
ACE inhibitors		66.0	31.0	40.5	25.1	32.2
aspirin or antiplatelet agents		79.2	88.8	42.5	50.0	90.2
beta blockers		46.4	91.0	58.0	26.8	78.2
calcium channel blockers		37.0		58.0		22.4
diuretics		26.3	17.8			10.0
lipid lowering drugs		60.1	81.0	49.0	34.1	88.4
multivitamins	22.5	11.6	28.8		22.9	

Table 1.6. Medication usage of participants in homocysteine lowering trials. The table shows medications being taken by participants, in addition to any homocysteine lowering treatment. Medications other than those listed, including calcium channel blockers, ACE inhibitors and oral hypoglycaemic agents, were also used to different extents in different trials. there was no significant difference in medication usage of participants in the active groups compared to the placebo groups.

1.2.4.4. *Summary of Evidence for Homocysteine as a Vascular Risk Factor*

Overall there is a large body of evidence showing that homocysteine is associated with vascular disease but there is still substantial controversy as to whether it is merely a marker of disease or causative. Although intervention trials have demonstrated little benefit in lowering homocysteine with vitamin therapy the results of such trials must be interpreted with caution and further work is required to see if such treatment might have a role in primary care or for selected targeted populations (eg chronic kidney disease, SLE). Even if homocysteine proves to be only a marker of disease, further research to uncover why it is increased in various forms of vascular pathology may be of value.

1.2.5. Possible Mechanisms for the Atherogenic Effect of Homocysteine

A number of mechanisms for an atherogenic effect by homocysteine have been proposed though has yet been proven. These mechanisms have been reviewed[44,45,46] and include effects on coagulation, generation and damage by free radicals, promotion of inflammation, decreasing nitric oxide production and binding to proteins with a subsequent alteration of their function.

A number of studies have suggested that homocysteine may have a pro-coagulant effect by increasing platelet adhesion, inhibiting protein C activation and activating factor V[44,45]. Many of these studies have been criticised as they used extremely high, non-physiological concentrations of homocysteine and not all have been reproducible[44,45]. A study by Mohan et al[42] investigated the effects of homocysteine on platelet function at physiological concentrations and concluded that homocysteine causes platelet activation and hypercoagulabilty, with the effects becoming apparent at approximately 50 μmol/L (a concentration corresponding to moderate hyperhomocysteinaemia). In a further study on platelet function Signorello et al[47] also concluded that homocysteine stimulates platelet aggregation, and that this was due to dysregulation of nitric oxide production by homocysteine. The decrease in nitric oxide production occurred in a dose dependent

manner with homocysteine concentrations in the range 10 - 100 μmol/L. An important feature of this study was that the effect was unique to homocysteine and that similar experiments with methionine and cysteine produced no effect upon platelets. Homocysteine at physiological concentrations has also been shown to induce the expression of thrombokinase by monocytes in a dose dependent manner[48] potentially leading to a pro-coagulant state.

Homocysteine can readily auto-oxidise to form both homocystine and mixed disulphides and in doing so generates reactive oxygen species such as superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) which may then attack cell membranes, lipoproteins, and platelets[49,50], however a particular weakness in this being a mechanism for homocysteine causing vascular disease is that cysteine may also undergo similar oxidation reactions, is in much higher concentration than homocysteine in plasma (approximately 20-30 fold[51]) but has not been associated with vascular disease[51].

Homocysteine forms stable disulphide bonds with protein cysteine residues and shows a greater tendency to do so than other thiols such as cysteine and glutathione[46]. It has been shown, both in vitro and in vivo, that this 'homocysteinylation' of proteins may lead to an impairment of protein function[46] with the suggestion that this may account for the possible atherogenic effects of homocysteine.

In cultured human aortic endothelial cells homocysteine has been shown to upregulate the production of interleukin-8 and monocyte chemoattractant protein-1 and may therefore contribute towards the initiation and progression of vascular disease by promoting leukocyte recruitment[52].

It has been shown that homocysteine may decrease the production of nitric oxide in the endothelium by increasing the concentration of asymmetric dimethylarginine (ADMA), a nitric oxide synthase inhibitor. This potentially leads to an increase in vascular stiffness, rendering the vasculature more susceptible to damage. This will be discussed in detail below.

1.2.6. Methods for the Analysis of Plasma Homocysteine

1.2.6.1 *General Considerations*

The first cases of homocystinuria were identified using simple screening methods: analysis of urine using the reaction of homocysteine with sodium nitroprusside to form a coloured complex (a test more commonly used for identifying cystinuria) and also by using thin-layer chromatography. Quantitative analysis was provided by amino acid analysers (using ion-exchange chromatography and post-column derivatisation with ninhydrin) which had only been recently developed[7].

Since that time the increasing interest in homocysteine as a possible cardiovascular risk factor has necessitated the development of highly specific and precise quantitative methods with high-throughput capacity, this, together with the identification of thiol selective/specific reagents, capillary electrophoresis, automated immunoassay techniques and bench-top mass spectrometers, has led to the existence and publication of a vast number of methods for homocysteine analysis. Homocysteine methods have been reviewed extensively[53, 54, 55, 56, 57, 58, 59, 60] and interest in method development continues.

The majority of methods measure total homocysteine by utilising a reduction step to release homocysteine bound to proteins and from disulphides and a number of different reducing agents have been employed[53]. Dithiothreitol (DTT) and mercaptoethanol have been used most commonly but are not suitable for use with methods making use of thiol specific derivatising reagents since they react with and consume reagent[53]. Sodium borohydride has been used but the evolution of gas during the reduction process has been problematic in automating methods[53]. Tributyl-n-phosphine is an effective reducing agent, used in a number of HPLC assays, but due to being poorly soluble in aqueous solution it is necessary to dissolve it in a carrier solution such as dimethylformamide before use[53]. Tris(2-carboxyethyl)phosphine (TCEP) is more soluble in water, and does not consume

derivatising agent[53], but having been introduced comparatively recently it has only been used in a limited number of applications[53].

1.2.6.2. *Radioenzymic assays*

Methods for the measurement of homocysteine in plasma and urine have been described[61,62]. These make use of S-adenosyl-L-homocysteine hydrolase to form radiolabelled S-adenosyl homocysteine (SAH) from homocysteine and ^{14}C labelled adenosine. The adenosylhomocysteine is isolated by using HPLC and collecting fractions eluted from the column and then quantitated using scintillation counting. The sensitivity of the method was such that it allowed the determination of free homocysteine (by first removing plasma proteins by acid precipitation) as well as total homocysteine and was used to investigate the extent of protein binding by homocysteine. It was though extremely labour intensive and this, in addition to the special requirements necessary for handling radioisotopes, meant that it was unsuitable for processing large sample numbers or for use in a routine clinical setting.

1.2.6.3. *Amino Acid Analysers*

Ion-exchange chromatography with post-column derivatisation by ninhydrin is a technique commonly employed by dedicated amino acid analysers used for identifying and monitoring patients with inborn errors of amino acid metabolism. Briddon[63] demonstrated that the use of a reduction step by DTT prior to sample analysis by Biochrom 20 amino acid analyser (Pharmacia Biotech) enabled the determination of total cysteine and homocysteine without substantially increasing analysis time or compromising the quality of analysis of other amino acids. Although this is useful for laboratories equipped with amino acid analysers such equipment is not common, generally being found only in centres offering a specialised amino acid referral service.

1.2.6.4. *HPLC*

Since homocysteine shows little native absorbance the majority of HPLC methods employ pre-column derivatisation for analysis. Traditional derivatising agents such as OPA react with all primary amino acids and so necessitate complex chromatography capable of separating the derivatised homocysteine from the other derivatised amino acids[57], and this has led to the development of thiol selective/specific reagents[64, 65.] A number of methods have been described making use of derivatisation with monobromobimane[66, 67, 68, 69,] a compound with little native fluorescence that reacts quickly with, and shows a high degree of selectivity, although not specificity, for thiols. Monobromobimane and thiols derivatised with it form fluorescent breakdown products upon storage which can complicate chromatographic separation of the compounds of interest[54]. The derivatising agent that has proved most popular is 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F), a reagent which shows no native fluorescence, exhibits a high degree of specificity towards thiols[57], and has led to the development of a number of highly sensitive methods[70, 71, 72 73]. The breakdown products of SBD-F and its derivatives, formed upon storage, are non-fluorescent which results in chromatograms with well-resolved analyte peaks free from interfering reagent peaks[57]. The main disadvantage of SBD-F is considered to be the relatively long reaction time for full derivatisation of thiols (1 hour at $60^{O}C$)[57]. HPLC with electrochemical detection has been used for the routine analysis of biological thiols[74, 75, 76,] and has the major advantage that derivatisation of thiols is not necessary. The methods described show high specificity and sensitivity but precision is poorer than those methods employing fluorometric detection[54].

1.2.6.5. *Gas Chromatography Mass Spectrometry.*

Methods utilising GS-MS have been developed[77, 78, 79] but have not found widespread use, in part because the methods are considered cumbersome[54]. With GC-MS it is possible to use an isotopically labelled form of homocystine as internal standard, resulting in methods with high precision and accuracy.

1.2.6.6. *Capillary Electrophoresis*.

A number of methods for homocysteine utilising capillary electrophoresis have been published[80,81,82] and these methods generally have very short run times. Although photometric detection of thiols, after derivatisation with reagents such as 5,5'-dithio-bis-2-nitrobenzoic acid (Ellman's reagent), is possible, in order to gain sufficient sensitivity and precision for the determination of homocysteine in biological fluids, either electrochemical detection is required or laser induced fluorescent detection following thiol derivatisation with reagents such as fluorescein isothiocyanate (FITC) or SBD-F[60]. Capillary electrophoresis equipment is not commonly found in routine clinical laboratories and these methods have not found widespread adoption.

1.2.6.7. *Immunoassay*

A number of commercially available immunoassays utilising enzyme-linked immunoassay (EIA)[83], chemiluminescence immunoassay (ICL)[84] or fluorescence polarisation immunoassay (FPIA)[85] for plasma homocysteine measurements are available. All the immunoassays developed so far share a common feature in that, following a reduction step, homocysteine is converted by S-adenosyl-L-homocysteine hydrolase to S-adenosylhomocysteine, which then reacts with an anti-SAH antibody. An EIA is available from Axis Biochemicals, ICL is used on the Immulite 2000 analyser (DPC) and FPIA was used on the IMx® analyser (Abbot laboratories). Although support for the latter instrument has now been withdrawn (2009) the FPIA is still available for use on the Architect® and AxSYM® analysers (Abbott Laboratories). ICL and FPIA provide automated means for determining plasma homocysteine with little manual intervention but they are considerably more expensive in terms of reagent cost per sample as compared to other methods (HPLC, MS/MS).

1.2.6.8. *Enzymatic Analysis*

Plasma homocysteine can be analysed by making use of enzymatic analysis. In a method described by Tan et al[86], following reduction step using DTT, a recombinantly produced homocysteine α, γ-lyase[87] is used to covert homocysteine to α-ketobutyrate, ammonia and H_2S. The H_2S then reacts with N,N-dibutylphenylene diamine to form a highly fluorescent product. The procedure was performed in microtiter plates, required only five microlitres of sample and compared well with an HPLC method utilising SBD-F derivatisation and fluorescent detection. The authors reported that cysteine showed a small degree of cross reactivity in the analysis but claimed that with the assay conditions used there was almost no interference by plasma cysteine when measuring homocysteine. An enzymatic cycling assay has been described and evaluated[88], [89]. In this method homocysteine reacts with S-adenosylmethionine (SAM) in the presence of a homocysteine S-methyltransferase to form methionine and S-adenosylhomocysteine (SAH); it should be noted that the SAH is formed by the loss of a methyl group from SAM and does not contain any part of the original homocysteine molecule. SAH is then hydrolysed into adenosine and homocysteine by SAH hydrolase. The adenosine is hydrolysed into inosine and ammonia, the latter then reacts with glutamate dehydrogenase, with an associated conversion of NADH to NAD and the reaction monitored by the decrease in absorbance at 340 nm. The homocysteine newly formed by the hydrolysis of SAH cycles back into the reaction providing a substantial amplification of the detection signal. This method is available commercially from Diazyme Laboratories (California, USA) and may be implemented on a number clinical chemistry analysers including the Roche Hitachi, Dade Dimension® and Beckman Synchron CX. Another commercially available cycling enzymatic method for plasma homocysteine, the performance characteristics of which have been evaluated[90, 91,] is that offered by Carolina Liquid Chemistries (Winston-Salem, North Carolina, USA). This uses cystathionine-β-synthase to convert homocysteine and serine to cystathionine, the latter is then converted by cystathionine-β-lyase to homocysteine, pyruvate and ammonia. The pyruvate then reacts with lactate dehydrogenase with an associated conversion of NADH to NAD and the reaction monitored by the decrease in absorbance at 340 nm. Applications of this method

are available for automated instruments including those produced by Dade, Beckman, Roche, Bayer and Olympus.

1.2.6.9. *Tandem MS*

In 1999 the first paper to describe the use of liquid chromatography tandem mass spectrometry (LC-MS/MS) for the measurement of plasma and urine homocysteine was published by Magera et al[92]. It employed very simple sample preparation (reduction using DTT for 15 min followed by protein precipitation using acetonitrile/formic acid/trifluoroacetic acid) and also used isotopically labelled homocystine for internal standardisation. The analysis time by the mass spectrometer was fast at 3 min per sample. The precision and recovery studies performed were not very thorough, both being performed using bovine calf serum (diluted 1:1 with distilled water) which had been spiked with homocysteine standard; only six replicates of each preparation were analysed to assess precision. Human plasma and urine were not used in these experiments. Nevertheless, results from MS/MS analysis compared favourably with those obtained by an HPLC assay (using derivatisation with monobromobimine and fluorescent detection) and with those obtained using FPIA on an Abbott IMx analyser. Since that initial paper a number of other similar methods have been published with adaptations to allow for the simultaneous determination of other compounds such as cysteine, methionine, folate and methylmalonic acid and with application to other matrices such as dried blood spots and intracellular fluid[93, 94, 95, 96, 97, 98, 99, 100, 101, 102].

Tandem mass spectrometry is often referred to as being an expensive technique, at least in terms of capital outlay, but as the cost of mass spectrometry analysers has fallen in the last few years such equipment is being found more commonly in large routine clinical laboratories and where this equipment is already in place its use provides a very economical means of homocysteine analysis as regards consumables. Even though isotopically labelled internal standards may seem expensive, a small quantity is sufficient for many analyses (eg 0.1g homocysteine-d_8 costs approximately £600 [2010 prices], but is sufficient for in excess of one million analyses). The use of isotopically labelled internal standards makes MS/MS a candidate reference method for homocysteine analysis[100], this with the potential

for multi-analyte determination, simple sample preparation and reasonably high sample throughput makes it a good choice for plasma homocysteine measurements in laboratories already employing tandem mass spectrometry.

1.3. **Methylated Arginines**

1.3.1. The Biochemistry and Metabolism of Methylated Arginines

Asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) and monomethylarginine (MMA) are naturally occurring amino acids that circulate in plasma and are excreted in the urine. They are formed by the continual enzymatic methylation of arginine residues within proteins by protein methyl transferases (PRMT)[103] and their structures are shown, together with that of arginine, in figure 1.4.

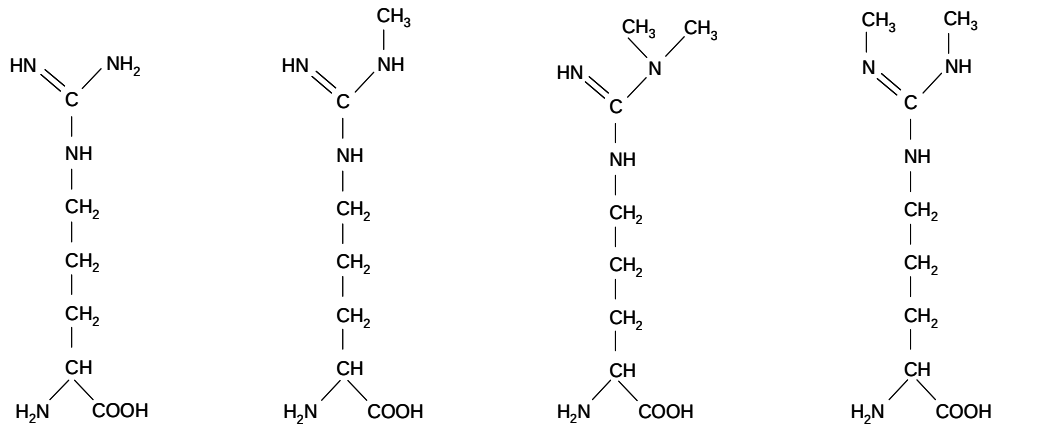


Figure 1.4. The structures of arginine and methylated arginines.

A number of PRMTs have been identified and fall into two classes. PRMT 1 form MMA and ADMA, whilst PRMT 2 form MMA and SDMA[103]. Upon proteolysis these methylated arginines are released into cells and subsequently into the circulation[103] from which they may then be actively transported into other cells by a y^+ cationic amino acid transporter protein[104]. Approximately 300 μmol ADMA is generated per day by humans[105], around 90% of which is metabolised by the enzyme dimethylarginine dimethylaminohydrolase (DDAH)[106], of which there are two isoforms (DDAH 1 and DDAH 2), and the remainder is excreted unchanged via the kidney. MMA is also metabolised by DDAH whereas SDMA is not[107], and it is thought that the only route of elimination of the latter is via the kidney.

1.3.2. Methylated Arginines, DDAH, and Nitric Oxide

ADMA and MMA are natural inhibitors of nitric oxide synthases (NOS) with ADMA being a more potent inhibitor of endothelial NOS (eNOS) and neuronal NOS (nNOS) than of inducible NOS (iNOS)[108]. SDMA has been shown to have no effect upon these enzymes[109]. NOS act upon arginine to produce nitric oxide (NO) and citrulline. The resultant NO induces vascular relaxation and also inhibits platelet adhesion and smooth muscle proliferation[110].

DDAH and NOS isoforms show distinct, and related, patterns of expression in different tissues[111]. DDAH 1 predominates in tissues that express nNOS (brain, kidney) whilst DDAH 2 predominates in endothelial cells and highly vascularised tissues that express eNOS (heart, placenta, kidney) and in immune tissues that express iNOS (spleen, thymus, bone marrow)[111,112]. Distribution of NOS and DDAH isoforms in the kidney is complicated, eNOS having been demonstrated in microvascular endothelial cells and the ascending loop of Henle, nNOS in the Bowman's capsule and collecting ducts and iNOS in the proximal tubule, ascending loop of Henle and distal convoluted tubules whilst DDAH 1 has been identified in the proximal convoluted tubule and DDAH 2 in the ascending loop of Henle and distal convoluted tubule[108].

In a study designed to investigate DDAH isoform expression on plasma ADMA concentrations, rats were administered an RNA inhibitor specific for either DDAH 1 or DDAH 2[113]. In a control group, DDAH 1 in the kidney and liver were shown to be two and seven fold higher, respectively, than DDAH 2 whereas DDAH 2 was five fold higher than DDAH 1 in mesenteric resistance vessels. Following inhibition of DDAH 1, a 35-50% decrease in its expression was observed in kidney and liver (with no significant change in DDAH 2) after 72 hours, with an associated 25% increase in plasma ADMA. Selective inhibition of DDAH 2 resulted in a 40-85% decrease in its expression (with no change in DDAH 1) but in this case no increase in plasma ADMA was observed. The authors concluded that plasma ADMA concentrations are regulated by the DDAH 1 isoforms whereas intracellular ADMA concentrations (and therefore production of NO) is primarily regulated by the DDAH 2 isoform. This conclusion is consistent with another study in which the liver has been shown to be a major organ for the elimination of circulating ADMA[114].

In vitro studies have shown that DDAH 2 (but not DDAH 1) expression is up regulated by NO, which possibly provides a positive feedback loop that serves to maintain endothelial NO concentration[115]. Other studies have shown that nitrosylation of DDAH 1 by NO leads to a reduction in its activity (DDAH 2 was not investigated)[116].

The growing body of evidence regarding the relationships between NO, ADMA and DDAH have now led to the suggestion that ADMA is a regulator of NO production, controlled by DDAH activity, and that pathological decreases in DDAH activity may lead to an increased ADMA concentration, a decreased production of NO, and thereby a pro-atherogenic state[108]. Although SDMA has no direct inhibitory effect upon nitric oxide synthase it has been suggested that it may compete with arginine for entry into cells, and therefore increased plasma SDMA concentrations may limit production of nitric oxide by limiting the availability of arginine[117] (figure 1.5).

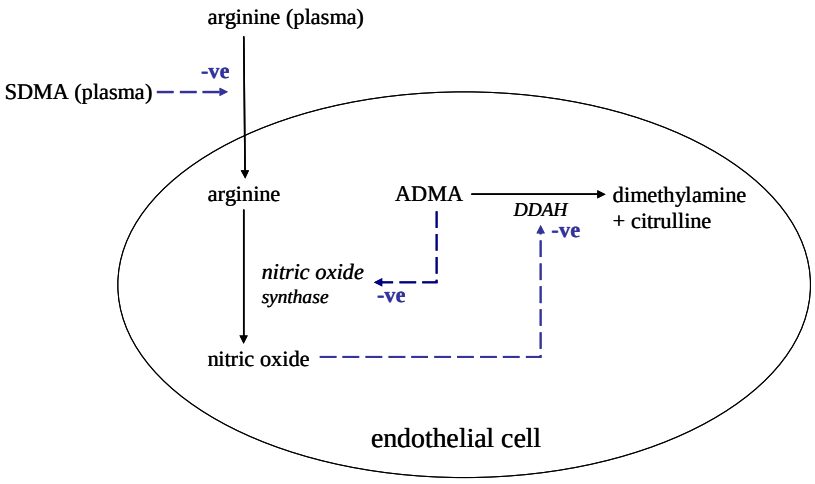


Figure 1.5. The effects of ADMA and SDMA on nitric oxide production. ADMA directly inhibits nitric oxide synthase and NO production. It has been suggested that SDMA may compete with arginine for entry into cells[117] and thereby also decrease NO synthesis.

1.3.3. Inhibition of DDAH

The active site of DDAH consists of a catalytic triad (cys 249, his 162, glu 114)[107] and it has been shown by Stühlinger et al that homocysteine may bind to this site with a subsequent loss of enzyme activity[118]. In the experiments they described: i) cultured bovine aortic endothelial cells were incubated with methionine or homocysteine (at physiological concentrations) and resulted in a dose dependent increase in ADMA production which was attenuated by the addition of the antioxidant pyrrrollidine dithiocarbamate (PDTC). ii) the activity of recombinantly produced DDAH was decreased following incubation with homocysteine, but not by methionine or cysteine, and the reduction in activity associated with homocysteine was attenuated in the presence of both PDTC and DTT. iii) DDAH was incubated with biotinylated homocysteine, applied to a streptavidin coated polystyrene plate and after washing DDAH was detected using a primary anti-DDAH antibody and peroxidase labelled secondary antibody; homocysteine was to have bound significantly to DDAH but similar experiments with biotinylated methionine and cysteine showed that their was no binding by these two amino acids. The essential points of these experiments were that they demonstrated that physiological concentrations of homocysteine resulted in an increased production of ADMA but that no effect was observed with cysteine.

A reduction in DDAH activity with increased production of ADMA has also been shown in cells cultured with oxidised LDL (but no effect with native LDL)[119], cells cultured with cigarette smoke extract[120], in cells cultured with 25.5 mmol/l glucose (the effects of which could be reversed by the antioxidant polyethylene glycol-conjugated superoxide dismutase)[121], and in rats with streptozotocin induced diabetes mellitus[121].

1.3.4. ADMA and Clinical Studies

A small number of studies have been performed examining the effects of ADMA infusion in humans[105,122,123,124,125] and these have been reviewed[126]. The reported effects include decreases in forearm blood flow, renal plasma flow, cardiac output and heart rate and increases in systemic vascular resistance and mean arterial blood pressure; the results of these studies are summarised in table 1.6.

Reference	Subjects (n)	Application	Dose	ADMA plasma concentration	Effect
Vallance et al (1992)	5	i.a. bolus	8 μmol	not reported	Decrease in forearm blood flow by 28%
Achan et al (2003)	6	i.v.	3.0 mg/kg	2.6 μmol/L (30 min post injection)	Decrease in heart rate by 9% and cardiac output by 15%. Increase in mean arterial blood pressure by 6% and systemic vascular resistance by 24%
Kielstein et al (2004)	6	i.v infusion over 40 min	0.5 - 10.0 mg/kg	4.2 - 42.1 μmol/L	Dose-dependent decrease in effective renal plasma flow of up to 11%
Kielstein et al (2004)	7	i.v infusion over 40 min	4.0 mg/kg	23.0 μmol/L	Decrease in cardiac output by 14%, increase in systemic vascular resistance by 11%
Kielstein et al (2004)	12	i.v infusion over 40 min	0.8 mg/kg	not reported	Decrease in effective renal plasma flow by 10%. Increase in filtration fraction by 17%
Kielstein et al (2005)	7	i.v infusion over 40 min	0.1 mg/kg	not reported	Increase in pulmonary vascular resistance by 38%

Table 1.7. Summary of studies investigating the phyiological effects of ADMA infusion. Adapted from Kielstein et al[126].

A major criticism of these studies is that following infusion, plasma ADMA concentrations, where they have been measured, are often much greater than those found in health or disease states (typical 'healthy values 0.4 - 0.6 μmol/L, pathological values 2.6 - 5.3 mmol/L)[127] and that they are not therefore representative of the effects of plasma ADMA found at physiological concentration. It has been suggested that the infusion studies are still valid, since they possibly represent the intracellular concentration of ADMA, which may be considerably higher than plasma[127]. This argument though would not seem to be valid: in studies using cultured bovine aortic endothelial cells, incubation with 10 μmol/L ADMA (in the presence of 100 μmol/L arginine) resulted in intracellular ADMA concentrations increasing from 2.7 μmol/L to 23.5 μmol/L over a twenty minute period[128]. It might be expected, therefore, that in the infusion studies, most of which administered ADMA over a 40 minute period, there might also be a substantial increase in intracellular ADMA concentrations as well as plasma concentrations and that these are not representative of those occurring naturally. Unfortunately it is not possible to address this point further as intracellular concentrations of ADMA were not determined in any of the studies.

Increased plasma ADMA concentrations have been shown to be associated with impaired glycaemic control. Abbasi et et al demonstrated that ADMA is significantly increased in patients with type 2 diabetes mellitus (mean ADMA: controls (n=18), 0.69 ± 0.04 μmol/L, type 2 diabetics (n = 16), 1.59 ± 0.22 μmol/L)[129] whilst Anderson et al investigated 355 patients with normal glycaemic control (but undergoing investigation for coronary artery disease), 239 patients with impaired fasting glucose and 256 patients with overt diabetes and found that ADMA increased with loss of glycaemic control[130]. Stühlinger et al determined the insulin sensitivity status of 64 non-diabetic, healthy volunteers and found that ADMA concentrations were significantly higher in those individuals classed as insulin resistant compared to those with normal insulin sensitivity. Furthermore, for patients with insulin resistance, treatment with rosiglitazone to increase insulin sensitivity decreased the plasma ADMA concentration[131].

A prospective study of 1874 patients with coronary artery disease and a median follow up of 2.6 years showed that ADMA and B natriuretic peptide were the strongest predictors of

death from cardiovascular cause or non-fatal myocardial infarction, independent of traditional cardiovascular risk factors[132]. A one year follow up of patients with advanced peripheral artery disease showed that plasma ADMA concentrations predicted major adverse cardiovascular events[133]. In a multicenter study ADMA and traditional cardiovascular risk factors were determined in 131 patients with coronary heart disease (CHD) and 131 controls (matched for age, sex and BMI), and the researchers concluded that ADMA was an independent risk factor for CHD[134]. In a further prospective study 2543 individuals with and 695 without coronary artery disease were investigated with a median follow up of 5.5 years. Plasma ADMA concentrations were found to be a predictor of all-cause and cardiovascular mortality in individuals with coronary artery disease and, other than showing a strong association with plasma homocysteine and glomerular filtration rate, was independent of established risk factors[135].

A study of 225 patients with end-stage renal disease (ESRD), receiving haemodialysis three times per week, has shown that plasma ADMA is strong, independent predictor of overall mortality and cardiovascular events[136]. In addition ADMA has been shown to independently predict progression to ESRD and mortality in patients with chronic kidney disease[137] and the higher the baseline ADMA concentration the faster the progression[138].

Clinical studies in which both plasma ADMA and plasma homocysteine have been measured are conflicting regarding the association between the two. Positive associations have been reported in patients with Alzheimer's disease[139], patients being investigated for chest pain[140] and patients having suffered a stroke[141] whereas other studies involving patients with ESRD[136] and patients undergoing percutaneous coronary intervention[142] have shown no association between the two.

1.3.5. Methods for the Measurement of ADMA

The measurement of dimethylated arginines in plasma presents a particularly difficult analytical challenge and a number of reviews of methods for their determination have been published[143,144,145,146]. A summary of methods including sample preparation and internal standard used is given in table 1.7.

Being structural isomers, ADMA and SDMA are difficult to separate chromatographically and also produce very similar fragmentation patterns with tandem mass spectrometry. The majority of published methods employ HPLC and, as ADMA and SDMA are both present in plasma at low concentration (approximately 1 μmol/L) and show little native absorbance, these methods have required the use of pre-column derivatisation. No specific derivatisation reagent for ADMA or SDMA has been reported and so derivatisation is usually performed with general amino acid derivatising reagents, OPA having been the most popular choice[147,148,149,150,151], although naphthalene-2,3-dicarboxaldehyde (NDA)[152], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor™)[153] and ninhydrin[154] have also been used. These methods generally have long run times and require gradient elution to separate ADMA from SDMA and from other derivatised amino acids; complete resolution of ADMA, SDMA and/or internal standard has not always been achieved.

The similarity in the fragmentation patterns of ADMA and SDMA produced by tandem mass spectrometry has meant that the majority of methods developed using this technique have also relied upon chromatographic separation of the two isomers, prior to mass spectrometric analysis, usually by HPLC[155,156,157,158], although GC[159,160] has been described. In GC methods, derivatisation of the amino acids is used primarily to increase their volatility, whereas with HPLC methods derivatisation, most commonly with OPA, is frequently used to facilitate the chromatographic separation of the two isomers, which is again not always completely achieved. A few tandem mass spectrometric methods have been described which have identified and made use of specific fragments for ADMA (and sometimes SDMA) without derivatisation[161,162,163], and also following butylation[164,165], of the amino acids.

Reference	Technique	Analytes Measured	Sample Preparation	Internal Standard	Comments
Petterson et al (1997)[147]	HPLC	ADMA, SDMA	cation exchange SPE, OPA derivatisation	none	Gradient elution, 70 min run time, ADMA and SDMA barely separated, requires 1.5 ml plasma
Chen et al (1997)[148]	HPLC	ADMA	protein precipitation (SSA), centrifugation, OPA derivatisation	none	Gradient elution, 35 min run time, SDMA not investigated, no evidence that SDMA is separated from, and does not interfere with ADMA
Teerlink et al (2002)[149]	HPLC	ADMA, SDMA, arg, Harg	cation exchange SPE, OPA derivatisation	MMA	Isocratic, 20 min run time, ADMA and SDMA just separated
Marra et al (2003)[152]	HPLC	ADMA, SDMA, arg	cation exchange SPE, NDA derivatisation	N^{ω}-propyl-L-arginine	Isocratic, 30 min run time, excellent separation of ADMA and SDMA
Zhang et al (2004)[150]	HPLC	ADMA, SDMA, arg, MMA, citrulline, ornithine, agmatine, hydroxy-L-arginine	protein precipitation (ethanol), centrifugation, lyophilisation, OPA derivatisation	Harg	Gradient elution, 40 min run time, ADMA and SDMA just separated
Heresztyn et al (2004)[153]	HPLC	ADMA, SDMA, arg	protein precipitation (SSA), cation exchange SPE, derivatisation with AccQ-Fluor™	MMA	Two extractions required 1) for ADMA & SDMA, 2) for arg. Gradient elution, run time approximately 40 min. Excellent separation of ADMA and SDMA but poor separation of internal standard from Harg.
Sotgia et al (2008)[154]	HPLC	ADMA	protein precipitation (acetonitrile), centrifugation, ninhydrin derivatisation, incubation with H_2SO_4 at 100 ^{O}C	Harg	Isocratic with 12 min run time. SDMA did not derivatise using the procedure described and was not detected. Good separation of ADMA form other peaks but poor separation of internal standard (Harg).
Blackwell et al (2009)[151]	HPLC	ADMA, SDMA, arg, Harg	cation exchange SPE, OPA derivatisation	monoethylarginine	Isocratic, 35 min run time, good separation of ADMA and SDMA.
Tsikas et al (2003)[159]	GC-MS/MS	ADMA	ultrafiltration, methylation (MeOH), derivatisation with PFPA/ethyl acetate	ADMA methylated with CD_3OD	Approximately 15 min run time. sample and internal standard are not treated identically (separate methylation procedures).
Albsmeier et al (2004)[160]	GC-MS	ADMA	protein precipitation (acetone), derivatisation with PFPA/methanol	ADMA-d_6 *	Approximately 9 min run time. Method applied to cell cultures as well as plasma.
Huang et al (2003)[155]	LC-MS, APCI	ADMA, SDMA. arg	protein precipitation (SSA), centrifugation	Harg	isocratic, run time 5 minutes. Poor separation of ADMA and SDMA. Method applied to urine measurements but not plasma.

Table 1.8. Methods for the analysis of plasma ADMA,
continued on following page

Reference	Technique	Analytes Measured	Sample Preparation	Internal Standard	Comments
Martens-Lobenhoffer et al (2004)[156]	LC-MS, ESI	ADMA, SDMA, arg	protein precipitation (acetonitrile), OPA derivatisation	ADMA-d_6 *, Harg, $^{13}C_6$-arg	Gradient elution, 20 min run time. ADMA and SDMA fairly well separated.
Martens-Lobenhoffer et al (2006)[158]	LC-MS, ESI	ADMA, SDMA, arg	protein precipitation (acetonitrile), OPA derivatisation	ADMA-d_6 *, homoarginine, $^{13}C_6$-arginine	Gradient elution, 20 min run time. . ADMA and SDMA separated to baseline.
Vishwanathan et al (2000)[161]	LC-MS/MS, ESI	ADMA, SDMA, MMA, arg	protein precipitation (acetonitrile/TCA)	$^{13}C_6$-arg	Isocratic, 15 minute run time. Specific fragments used for monitoring ADMA (203 → 158) and SDMA (203 → 172).
Kirchherr et al (2005)[157]	LC-MS/MS, ESI	ADMA, SDMA	protein precipitation (acetonitrile/methanol), centrifugation	leucine-d_3	Gradient elution, 6 min run time. ADMA and SDMA separated to baseline.
Schwedhelm et al (2005)[164]	LC-MS/MS, ESI	ADMA, SDMA, arg	protein precipitation (acetone), derivatisation with butanol/HCl	arginine-d_7, ADMA-d_6 *	Gradient elution, run time 4 min. Specific fragments used: ADMA (259 → 214), SDMA (259 → 228).
Martens-Lobenhoffer et al (2006)[162]	LC-MS/MS, ESI	ADMA, SDMA, arg	protein precipitation (acetonitrile/TFA/propionic acid), centrifugation	ADMA-d_6 * $^{13}C_6$-arginine	Isocratic, run time 6 min. Specific fragments used for monitoring ADMA (203 → 46) and SDMA (203 → 172).
Schwedhelm et al (2007)[165]	LC-MS/MS, ESI	ADMA, SDMA. arg	Protein precipitation (methanol), derivatisation with butanol/HCl, performed in microtitre plates.	arginine-d_7, ADMA-d_6 *	Isocratic, run time 1.6 min. Specific fragments used for monitoring ADMA (259 → 214) and SDMA (259 → 228).
Bishop et al (2007)[163]	LC-MS/MS, ESI	ADMA, arg	ultrafiltration	lysine-d_4, $^{15}N_2$-arginine	Gradient elution, run time 2 min. ADMA determined by specific fragment (203 → 46).
Caussé et al (2000)[166]	CE	ADMA, arg	dilution of sample in water (1/1000), FITC derivatisation	Harg	Run time approximately 11 min. ADMA and internal standard not completely resolved.

Table 1.8. Methods for the analysis of plasma ADMA
continued from previous page

A method using capillary electrophoresis with laser-induced fluorescence has been published[166], samples being derivatised with fluoresceine isothiocyanate after precipitation of proteins with sulphosalicylic acid. The paper initially describes experiments with ADMA, SDMA and arginine, claiming good separation of the two dimethylarginines. Subsequent experiments and method evaluation were performed with modifications to the procedure in order to reduce analysis time; SDMA not being analysed. Inspection of the electropherograms published would suggest that with these modifications resolution of ADMA from the internal standard (Harg) was not complete.

An ELISA for plasma ADMA is commercially available (DLD Diagnostika GmbH, Hamburg, Germany). In this method plasma ADMA is first acylated and then competes with ADMA bound to a microtitre plate for a limited number of rabbit anti-ADMA antiserum binding sites. After equilibrium has been achieved the plate is washed and the antibody-ADMA complex bound to the plate reacted with a second, peroxidase labelled, antibody (goat anti-rabbit). Quantitation is performed using 3,3',5,5'-tetramethylbenzidine as peroxidase substrate. Evaluations of this method are conflicting. Valtonen et al[167] compared it with an HPLC method and reported that there was no correlation between the two, whilst Širok et al[168] in a similar comparison of ELISA with HPLC found a strong correlation between the two methods and claimed that the "HPLC method for ADMA determination can be replaced by ELISA which gives comparable results". It is likely that the latter group did not correctly interpret their data as one of the figures they presented shows that the values they obtained using ELISA were approximately double that found by HPLC (the regression equation given: ELISA ADMA = 2.303 x HPLC ADMA - 0.1622). In another comparison study results obtained by ELISA were approximately twice that obtained using LC-MS[169], the authors also noted that the results of the ELISA method seemed to be strongly influenced by differences in sample matrix.

In addition to the discrepancy in results obtained when using ELISA, it has also been reported that there is considerable variation in plasma ADMA concentrations obtained using different methods in general. In reviewing 41 published studies Horowitz and

Heresztyn[145] noted that the mean plasma ADMA concentration of normal subjects ranged from 0.30 - 4.02 μmol/L, but also stated that "the majority of MS-based assays suggest the mean value for ADMA in 'normal' plasma lies within the range 0.36 - 0.60 mmol/L".

The variation in results obtained using different methods has been attributed in part to poor chromatographic separation by some methods of ADMA and SDMA, and also due to the use of different sample preparation techniques coupled with a lack of appropriate internal standardisation[143]. Sample preparation techniques have included ultrafiltration, cation exchange solid phase extraction and protein precipitation using a variety of different agents including sulphosalicylic acid, acetonitrile, ethanol and acetone. The identification of suitable internal standards has been particularly problematic and some methods have not made use of internal standardisation. A number of methods have made use of either monomethylarginine or homoarginine, both of which are not ideal since they occur endogenously in plasma at variable concentration. Isotopically labelled ADMA has only become available relatively recently (2007), but some groups have synthesised their own based on a method described by Pundak and Wilchek[170] and subsequently modified by Albsmeier et al[160]. Isotopically labelled lysine and leucine have also been used as internal standards for the measurement of ADMA. Isotopically labelled SDMA is still not commercially available and its synthesis has not been described in methods for dimethylarginine determination. Chemical differences between an internal standard and an analyte may result in different behaviour between the two with regard to extraction, precipitation and derivatisation procedures and this is of particular importance when sample and standard matrix do not match. In addition analyte and internal standard may not ionise to the same extent in the mass spectrometer.

There is currently no agreed reference method for the measurement of dimethylated arginines, reference materials for their standardisation or quality assurance programmes and until these are established it is unlikely that agreement of plasma ADMA results produced by the various methods will be reached.

1.4. Arterial Stiffness

1.4.1. Overview

Although the heart delivers blood to the circulatory system in a pulsatile fashion the elastic nature of the arteries dampens the pulse and this, combined with the high resistance of the smaller diameter peripheral blood vessels, results in an almost smooth continuous flow of blood through the tissue bed[171].

The pulse wave velocity (PWV) is mainly dependent upon the elasticity of the arterial wall and is described by the Moens-Korteweg equation[172]:

$$c_0 = \sqrt{(Eh/2R\rho)}$$

where c_0 is the wave speed, E is Young's modulus in the circumferential direction, h is the wall thickness, R is the vessel radius and ρ is the density of fluid. From this it may be seen that PWV is proportional to the square root of the Young's modulus of elasticity of the blood vessel and, put simplistically, the stiffer the blood vessel the faster the pulse wave travels. The cellular and histological structure of arteries varies throughout the arterial tree, and also changes with age, the proximal arteries being more elastic than the distal[173,174].

In systole a pressure wave travels away from the heart through the arterial tree, this wave is reflected by bifurcation points creating a retrograde wave which travels back to the heart and, in young healthy individuals, reaches it in diastole. At any point in the arterial tree the blood pressure is due to the summation of pressures produced by the forward and reflected waves[175]. The fact that the retrograde wave reaches the heart in diastole is beneficial in that it enhances perfusion of the heart by the coronary arteries. In individuals with stiffer arteries the associated increase in pulse wave velocity results in the retrograde pulse wave reaching the heart in systole, rather than diastole, and increases the systolic pressure. The diastolic pressure is reduced resulting in impaired coronary perfusion[171].

Arterial stiffness has been shown to increase with age[176] and has also been shown to be a strong predictor, independent of other risk factors, for coronary events and stroke[177,178,179] and for mortality in both diabetes[180] and end-stage renal failure[181].

1.4.2. Measurements of Arterial Stiffness

Arterial stiffness can be measured regionally, systemically and locally[182]. In addition, analysis of the pulse waveform can be used to provide information about the nature of reflected waves. Carotid-femoral pulse wave velocity (CF-PWV), a non-invasive measure of regional arterial stiffness, has been described by a recent expert consensus document as being the 'gold standard' for determination of arterial stiffness[183] and has been used for the majority of clinical studies. With this method the pulse waveform is determined transcutaneously at both the right common carotid artery and at the right femoral artery; the waveform being recorded using pressure, distension or Doppler measurements. The foot of each waveform, defined as being the end of diastole, is identified and the difference in time between the two (Δt) is calculated. The distance, D, between the two sites is measured over the body surface, usually with a tape measure, and the CF-PWV calculated as $PWV = D/\Delta t$ (m/s). Some limitations to these measurements have been noted, the femoral waveform being sometimes difficult to record accurately in individuals with metabolic syndrome, diabetes and peripheral disease, and the distance between carotid and femoral sites may be inaccurately measured in those with abdominal obesity[182].

Systemic measures of arterial stiffness rely on theoretical modelling of the arterial system, based on comparisons to electrical circuits containing elements of capacitance and resistance. The number of theoretical approximations they make have limited their usefulness in being applied to clinical settings[183].

Local arterial stiffness is determined by measuring the difference in blood vessel diameter between diastole and systole. Ultrasound devices are commonly used for measurements on superficial arteries, particularly the carotid artery; measurements on deep arteries such as the aorta have also been performed using magnetic resonance imaging[182,183]. These

techniques generally require video image analysis, which limits the precision of the measurements made. High precision measurements of blood vessel diameter change can be made using echotracking devices. Local arterial stiffness measurements have the advantage in that they allow the determination of the Young's elastic modulus of the arterial wall but all the techniques suffer from the disadvantage in that they are more time consuming than measurements of pulse wave velocity and require a greater degree of technical expertise to perform[183]. In addition, although CF-PWV and locally determined carotid stiffness measurements general provide consistent information in healthy subjects, discrepancies have been noted for subjects with high blood pressure and/or diabetes[184]. Measurements of local arterial stiffness are therefore performed mostly for mechanistic rather than epidemiological studies.

Analysis of the *shape* of the arterial pressure waveform allows determination of the augmentation index (AIx) a parameter giving information regarding the reflected pulse wave[183]. As mentioned previously, with increased arterial stiffness the reflected pulse wave reaches the heart in systole and adds to (augments) the systolic, rather than diastolic, pressure. The resulting arterial pressure wave form shows a point of inflection corresponding to the initial (forward) pressure wave and a peak corresponding to the sum of forward and retrograde pressure waves. The augmentation index, as a percentage, is calculated as the ratio of pressure augmentation to pulse pressure (SP-DP).

Ideally the arterial pressure waveform should be measured at the ascending aorta to truly represent the load imposed upon the left ventricle but, as this is difficult practically, measurements are usually made at the radial artery and a mathematical transform used to convert them to those that which would be seen at the aorta[183]. Measurements can be made at the common carotid artery, being close to the aorta a mathematical transform does not need to be applied to the waveform obtained, but a higher degree of technical expertise is required for its measurement as compared to measurement at the radial artery. The AIx has been shown to be of all-cause and cardiovascular mortality in end stage renal disease[185] and

a predictor of cardiovascular events in patients undergoing percutaneous coronary interventions[186].

1.4.3. Homocysteine, and Arterial Stiffness

Conflicting results have been demonstrated by studies investigating whether vascular dysfunction, as determined by arterial stiffness measurements, is associated with plasma homocysteine concentrations[187,188,189,190,191,192] (table 1.8) however, these studies differed widely in the age of participants and the measures of arterial stiffness used. Also it should be noted that in all but one study[191], plasma homocysteine concentrations were only mildly elevated.

Reference	Patient category and sample numbers	Measures of arterial stiffness	Age (years)	Homocysteine (μmol/L)	Conclusions
Bortolotto et al 1999[187]	Hypertensive patients with and without clinical vascular disease, n = 236	CF-PWV	58 ± 13	no vascular disease: 13.6 ± 4.9, vascular disease present: 15.9 ± 5.3	CF-PWV positively correlated with plasma homocysteine
Woodside et al 2004[188]	Young healthy adults, males = 251, females = 238	CF-PWV, CR-PWV, CT-PWV	males: 22.4 ± 1.6, females 22.8 ± 1.7	males: geometric mean = 9.47 ± 1.43, females: geometric mean = 9.10 ± 1.43	no correlation between plasma homocysteine and any measure of PWV
Tso et al 2006[189]	Chinese females with systemic lupus erythematosus, n = 58	ba-PWV	40 ± 15	13.19 ± 6.10	ba-PWV positively correlated with plasma homocysteine for total patient group. When the group was subdivided according to the presence of anticardiolipin antibodies a positive correlation was observed for the antibody positive group but not the antibody negative group.
Levy et al 2007[190]	Members of the Framingham Study offspring Cohort. Individuals were excluded on the basis of prior myocardial infarction or heart failure. Males: n = 856, females n = 1106.	CF-PWV, CR-PWV	males: 60.5 ± 9.8, females 61.1 ± 9.5	Males: median = 8.5, IQR = 7.2 - 10.0, Females: median = 7.1, IQR = 5.9 - 8.8	Males: CF-PWV, but not CR-PWV positively correlated with plasma homocysteine (p = 0.036). For females there was no correlation between plasma homocysteine and either CF-PWV or CR-PWV).
Ertek et al 2009[191]	Peritoneal dialysis patients, n = 60	CF-PWV	46.2 ± 13.2	27.2 ± 15.7	No association found between plasma homocysteine and CF-PWV.
Vyssoulis et al 2010[192]	Normotensive controls, n = 31. Patients with isolated office hypertension, n = 480 and patients with essential arterial hypertension, n = 1188.	CF-PWV AIx	Normotensives: 45.8 ± 1.0, isolated office hypertension: 52.4 ± 0.6, arterial hypertension: 52.4 ± 0.3	Normotensives: 9.3 ± 0.2, isolated office hypertension: 10.5 ± 0.2, arterial hypertension: 11.7 ± 0.1	CF-PWV correlated positively with plasma homocysteine when all three groups were combined and for each of the three groups separately. AIx correlated positively with plasma homocysteine when all three groups were combined, for the patients with isolated office hypertension and for patients with arterial hypertension but for normotensive controls.

Table 1.9. Studies investigating homocysteine and measures of arterial stiffness.

2. Experimental

2.1. Materials

Homocysteine method

Dithiothrietol, D,L-homocystine, D,L-cystine, D,L methionine and HPLC grade acetonitrile were obtained from Sigma-Aldrich (Gillingham, Dorset, UK). D,L-homocystine-d_8 and D,L-methionine-d_3 were purchased from Promochem (Welwyn Garden City, Hertfordshire, UK). D,L-cystine-d_4 was obtained from CK Gas Products (Wokingham, Berkshire, UK). Autonorm Human Liquid Control, level one, was purchased from Bio-Stat Ltd (Stockport, Cheshire, UK). Chromatography column: 3.3cm, 4.6mm id Supelcosil LC-CN column (Sigma-Aldrich, Gillingham, Dorset, UK).

ADMA method

Arginine, N^G, N^G-dimethylarginine hydrochloride, N^G, $N^{G\prime}$-dimethyl-L-arginine di(p-hydroxyazobenzene-p´-sulfonate) salt, N^G-methyl-L-arginine acetate salt, copper carbonate, cyanogen bromide activated Sepharose, dimethylamine, dimethylsulphate, 1,2-dimethyl-2-thiopseudourea hydroiodide, 1,3-dimethyl-2-thiourea, N,N´-dimethylurea and Supelclean LC-SCX solid phase extraction tubes were purchased from Sigma-Aldrich (Dorset, UK). Formic acid and 0.1 M HCl were obtained from VWR (Leicestershire, UK). Ammonia solution (35%) and HPLC grade methanol were purchased from Fisher Scientific (Leicestershire, UK). L-arginine-d_7:HCl and L-ornithine-d_2:2HCl were supplied by CK Gas Products LTD (Hampshire, UK).

2.2. Reagents and Methods for Homocysteine Experiments

Standards

Plasma and urine standards were prepared by spiking commercially available controls with stock standard (500 μL stock standard to a final volume of 10 mL of control). Autonorm™ Human Liquid Control, level one was used for preparing plasma standards and Lyphocheck

Quantitative Urine Control for preparing urine standards. The added concentrations were 0, 100, 200, 400 and 800 μmol/L for cysteine and 0, 10, 20, 40 and 80 μmol/L for homocysteine and methionine. The actual concentrations of the working standards were determined by analysing each standard five times and calculating the endogenous concentration of the analytes by the standard additions principle. Standards were frozen at -20 ^{O}C until use.

Internal standard

The internal standard was prepared by dissolving cystine-d_4, homocystine-d_8 and methionine-d_3 in 20 mmol/L HCl, resulting in final concentrations of 1 mmol/L, 50 μmol/L and 100 μmol/L respectively, this was stored frozen at -20^{o}C until use. The reducing agent, dithiothreitol (200 mmol/L) in 0.1 mol/L NaOH, was prepared just before use. The protein precipitating agent was prepared by the addition of 100 μL formic acid and 50 μL trifluoroacetic acid to 100 mL of acetonitrile.

Sample preparation

Samples were prepared by adding 50 μL of sample to 50 μL of internal standard, followed by 50 μL of reducing agent and, after mixing, these were left to stand at room temperature for 15 minutes. Protein precipitating agent (500 μL) was then added and, after mixing, the samples were centrifuged at 9000*g* for two minutes at which point the supernatant was removed and transferred to autosampler vials.

Mass spectrometric analysis

Ten microlitres of prepared sample was injected for analysis. The mobile phase consisted of acetonitrile:water (50:50 vol/vol) containing 0.1% formic acid and was pumped at a flow rate of 500 μL/min. The total analysis time was two minutes. The gas settings used with the mass spectrometer were, curtain gas (air) 40 psi, ion source gas 1 (nitrogen) 40 psi, ion source gas 2 (nitrogen) 60 psi and collision gas (nitrogen) 3 psi. The electrospray voltage was set to +5000 V and the nebuliser temperature to 500^{O}C. The parent/daughter transitions used to monitor each analyte and the associated potential settings are given in table 2.1; a dwell time of 100 ms was used for each transition.

	Parent ion (m/z)	Daughter ion (m/z)	Declustering potential (V)	Focusing potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell entrance potential (V)	Collision cell exit potential (V)
homocysteine	136.0	90.1	26	50	7.5	17	6	4
homocysteine-d_4	140.1	94.1	26	50	7.5	17	6	4
cysteine	122.0	76.0	11	360	7.5	19	6	4
cysteine-d_2	126.0	80.0	11	360	7.5	19	6	4
methionine	150.0	103.8	11	360	6	15	6	16
methionine-d_3	153.0	107.1	11	360	6	15	6	16

Table 2.1. Mass spectrometer settings for the analysis of homocysteine, cysteine and methionine.

Plasma methionine

This was determined using an in-house HPLC method[193]. Plasma proteins were precipitated with ethanol (twice) and amino acids were then derivatised with PITC. The derivatives were separated on a C18 column using gradient elution and the derivatised amino acids detected by absorbance at 254 nm. Norleucine was used as internal standard.

Urine cystine

The method used for the determination of urine cystine was that used routinely in the Clinical Chemistry and Immunology Department at the Royal Sussex County Hospital, Brighton. Four mL of water (blank), standard (420 μmol/L cystine) or urine was added to 1 g Amberlite IR-120(H) resin and mixed by inversion for five minutes, after which the liquid was removed from the resin and discarded. The resin was washed by adding four mL deionised water and mixing by inversion for five minutes. The water was removed and discarded. Three mL 3 mol/L ammonium hydroxide was added to the resin, and after mixing by inversion for a further five minutes, 1 mL of the ammonia solution was removed, transferred to a 1 mL plastic microcuvette and 500 μL of sodium cyanide (5% wt/vol) added. After fifteen minutes the absorbance of each solution was measured at 520 nm (abs1), 50 μL of sodium nitroprusside was added (5% wt/vol) and the absorbance at 520 nm measured exactly 10 seconds later (abs2). The change in absorbance Δabs (= abs2-abs1) was calculated for each solution and the concentration calculated according to the following formula:

$$\text{concentration in sample} = \frac{(\Delta\text{abs sample} - \Delta\text{abs blank})}{(\Delta\text{abs standard} - \Delta\text{abs blank})} \times \text{concentration of standard}$$

2.3. Reagents and Methods for Methylated Arginine Experiments

Stock standards.

ADMA (10 mmol/L): 27.5 mg ADMA.2HCl dissolved in 10 mL 0.1 mol/L HCl.

SDMA (10 mmol/L): 22.8 mg SDMA.di(p-hydroxyazobenzene-p'-sulphonate) salt dissolved in 3 mL 0.1 mol/L HCl.

MMA (10 mmol/L): 24.8 mg MMA acetate dissolved in 10 mL 0.1 mol/L HCl.

arginine (100 mmol/L): 870 mg arginine dissolved in 50 mL 0.1 mol/L HCl.

Working Standards.

For routine sample analysis four working standards were prepared by diluting stock solutions of ADMA, SDMA, MMA and arginine in 0.1 mol/L HCl and freezing portions of the standards at -20^{o}C until used.

The concentration of the working standards were:

standard A 0.5 μmol/L ADMA, SDMA and MMA, 20 μmol/L arginine

standard B 1.0 μmo/L ADMA, SDMA and MMA, 40 μmol/L arginine

standard C 2.0 μmol/L ADMA, SDMA and MMA, 80 μmol/L arginine

standard D 5.0 μmol/L ADMA, SDMA and MMA, 200 μmol/L arginine

These values were considered suitable for analysis of plasma samples but the literature suggested that the concentration of ADMA and SDMA in urine would be considerably higher and so in all subsequent analyses urine samples were pre-diluted 1 in 10 in deionised water.

Derivatising Agents.

a) Borate buffer, 200 mmol/L: 2.473 g boric acid was dissolved in approximately 180 mL deionised water and 5 mol/L sodium hydroxide then added to adjust the pH to 9.5. The solution was made up to a final volume of 200 mL with deionised water.

b) OPA (35 mmol/L) / mercaptoethanol (75 mmol/L): 25 mg OPA was dissolved in 500 µL methanol. Borate buffer, 4.5 ml, was then added followed by 26 µL mercaptoethanol.

c) OPA (35 mmol/L) / mercaptopropionylglycine (75 mmol/L): 25 mg OPA and 61.2 mg mercaptopropionyl glycine were dissolved in 500 µl methanol and 4.5 ml borate buffer then added.

d) OPA (35 mmol/L) / 3-mercaptopropanol (75 mmol/L): 25 mg OPA was dissolved in 500 µl methanol. Borate buffer, 4.5 ml, was then added followed by 32.5 µL mercaptopropanol.

e) OPA (35 mmol/L) / monothioglycerol (75 mmol/L): 25 mg OPA was dissolved in 500 µL methanol. Borate buffer, 4.5 ml, was then added followed by 31 µL monothioglycerol.

f) OPA (37 mmol/L) / 11-mercapto-1-undecanol (75 mmol/L): 27.5 mg OPA and 80 mg 11-mercapto-1-undecanol were dissolved in 4 mL propan-2-ol and 1 mL acetonitrile and 200 µL triethylamine then added.

g) NDA (37 mmol/L) / mercaptoethanol (75 mmol/L): 7.1 mg NDA was dissolved in 1 mL acetonitrile. Triethylamine, 40 µL, and mercaptoethanol, 5 µL, were then added.

NDA is very insoluble in aqueous solution and so the reagent preparation procedure that had been used for OPA reagents had to be modified.

h) NDA (37 mmol/L) / monothioglycerol (75 mmol/L): 7.1 mg NDA was dissolved in 1 mL acetonitrile. Triethylamine, 40 µL, and monothioglycerol, 5 µL, were then added.

i) PITC (40 mmol/L) : 5 μL PITC was added to 1 mL methanol and 40 μL triethylamine then added.

PITC is very insoluble in aqueous solution and so the reagent preparation procedure that had been used for OPA reagents had to be modified.

Derivatisation Procedures.

i) For reagents a) - f) and i) derivatisation of amino acids was carried out by adding 100 μL of derivatising reagent to 200 μL of standard. Derivatisation was allowed to proceed for at least five minutes before using the preparation.

ii) For reagents g) & h) derivatisation of amino acids was carried out by adding 100 μL acetonitrile to 100 μL standard followed by 100 μL derivatising reagent. Derivatisation was allowed to proceed for at least five minutes before using the preparation.

For the determination of MRM parameters 1 mL of derivatised standard was diluted in 9 mL 50:50 MeOH : 0.1 % formic acid

Synthesis of Isotopically Labelled ADMA, SDMA and MMA.

Isotopically labelled ADMA, SDMA and MMA was not commercially available when this work began, although isotopically labelled arginine (as arg-d_7) could be purchased commercially. Subsequently ADMA-d_7 has become commercially available from Cambridge isotope laboratories. A method for the synthesis of isotopically labelled ADMA from labelled ornithine has been described by Albsmeier[160], and this method was utilised here. Methods for the synthesis of SDMA[194], and MMA[195], had also been published and these were adapted to the synthesis of isotopically labelled internal standards.

An ornithine-d_2 copper complex was used as the starting point for synthesis of all internal standards. The preparation of the complex was based upon that described by Albsmeier et al[160].

a) For each synthesis an ornithine-d_2 copper complex was used as the starting point and this was prepared as follows: 20.6 mg ornithine-d_2:2HCl was dissolved in 1 mL water. Small amounts of copper carbonate were added, with mixing, resulting in the development of a blue colour. Copper carbonate continued to be added until saturation was achieved and the intensity of the colour no longer increased. The ornithine-d_2 copper complex preparation was centrifuged and the supernatant removed and stored at 4^{o}C for later conversion into ADMA-d_2, SDMA-d_2 or MMA-d_2.

b) Synthesis of ADMA-d_2: this was based upon the procedure described by Albsmeier et al[160]. In brief 0.5 g bromcyan-agarose was suspended in 10 mL of 1 mol/L HCl in methanol and mixed for 30 min. The bromcyan-agarose was then washed with 10 mL of 1 mmol/L HCl and three times with 10 mL water. The ornithine-d_2 complex prepared above (a) was added to the bromcyan-agarose and mixed overnight at 4^{o}C. The mixture was then centrifuged, the supernatant removed and discarded and the bromcyan-agarose washed with 10 mL 1 mol/L HCl and then three times with 10 mL water. The ornithine-d_2 bromcyan-agarose complex was suspended in 20 mL 20% dimethylamine and the mixture incubated at 50^{o}C for 24 hr. The supernatant was then removed and dried under vacuum, reconstituted in methanol: water 50:50, centrifuged and the supernatant again dried under vacuum. The residue was dissolved in 10 mL 0.1 mol/L HCl to yield a stock solution. Analysis of the product by mass spectrometry confirmed the formation of ADMA-d_2; no formation of ADMA, SDMA, MMA or arginine was detected.

c) Synthesis of SDMA-d_2: this was based upon a procedure described by Kakimoto and Akazawa[194] for the synthesis of SDMA from ornithine. Dimethylsulphate (50 µL) was added to dimethylthiopseudourea (54 mg). The container was sealed and heated to 110^{o}C for 30 min. The ornithine-d_2 copper complex prepared above (a) was added along with 100 µl 5M NaOH and 9 ml water. The mixture was then incubated at 30^{o}C for three days.

The mixture was centrifuged and the supernatant removed and dried under vacuum, reconstituted in methanol: water 50:50, centrifuged and the supernatant again dried under vacuum. The residue was dissolved in 10 ml 0.1 M HCl to yield a stock solution. Analysis of the product by mass spectrometry confirmed the formation of SDMA-d_2; no formation of ADMA, SDMA, MMA or arginine was detected.

d) synthesis of methylarginine-d_2: this was based upon a procedure described by Corbin and Reporter[195] for the synthesis of methylarginine from ornithine. To the ornithine-d_2 copper complex prepared above (a), 2 mL of 35% ammonia solution and 23.2 mg 1,2-dimethyl-2-thiopseudourea was added. The mixture was then incubated at 25^{o}C for 24 hr. The mixture was then centrifuged and the supernatant removed and dried under vacuum, reconstituted in methanol: water (50:50), centrifuged and the supernatant again dried under vacuum. The residue was dissolved in 10 mL 0.1 mol/L HCl to yield a stock solution. Analysis of the product by mass spectrometry confirmed the formation of MMA-d_2; no formation of ADMA, SDMA, MMA or arginine was detected.

Internal standard for routine use.

For routine analysis of plasma and urine samples a working internal standard solution was prepared by diluting the stock internal standard in 0.1 mol/L HCl. to give final concentrations of 20 μmol/L for ADMA-d_2, SDMA-d_2 and MMA-d_2 and 200 μmol/L for arginine-d_7.

Solid Phase Extraction

The extraction procedure was carried out by using a ten column solid phase extraction (SPE) manifold connected to a water pump. Samples were initially acidified by the addition of 1 mL of 0.1 mol/L HCl to 50 μL of working internal standard solution and 200 μL of plasma or urine sample; urines having been pre-diluted with deionised water (1 part urine: 9 parts water). Strong cation exchange solid phase extraction (SPE) columns were conditioned by washing with two reservoirs of methanol (approximately 1.2 mL each) followed by washing with two reservoirs of 0.1 mol/L HCl. The acidified samples were applied to the SPE columns and drawn through under reduced pressure with the flow rate

being maintained at approximately 1 mL/min. The SPE columns were then washed with two reservoirs of deionised water followed by two reservoirs of methanol. Analytes were then eluted from the SPE columns using 1 mL of ammonia/methanol (1 part 35% ammonia solution : 4 parts methanol), with this being drawn through the SPE columns under reduced pressure with the flow rate being maintained at approximately 1 mL/min. The eluates were dried under vacuum and then reconstituted in 1 mL of 0.1% formic acid.

Mass spectrometric analysis

Twenty microlitres of prepared sample was injected for analysis. The mobile phase consisted of 0.1% formic acid and was pumped at a flow rate of 500 µL/min. The total analysis time was three minutes. The gas settings used with the mass spectrometer were, curtain gas (air) 40 psi, ion source gas 1 (nitrogen) 10 psi, ion source gas 2 (nitrogen) 0 psi and collision gas (nitrogen) 4 psi. The electrospray voltage was set to +5000 V and the nebuliser temperature to 500^{O}C. The parent/daughter transitions used to monitor each analyte and the associated potential settings are given in table 2.2; a dwell time of 500 ms was used for each transition.

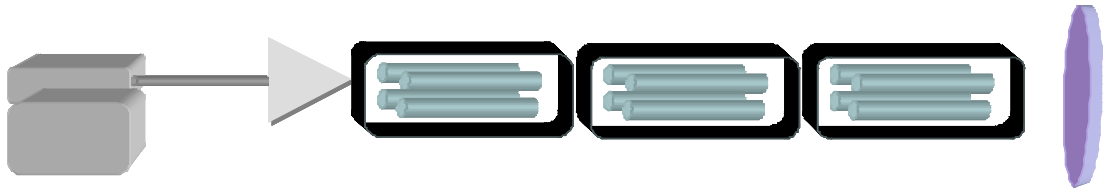
	Parent ion (m/z)	Daughter ion (m/z)	Declustering potential (V)	Focusing potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell entrance potential (V)	Collision cell exit potential (V)
ADMA	203.0	46.3	26	360	8	37	13.6	0
ADMA-d_2	205.0	46.3	26	360	8	37	13.6	0
SDMA	203.0	172.0	31	220	6.5	17	12.0	30
SDMA-d_2	205.0	174.0	31	220	6.5	17	12.0	30
MMA	189.0	70.1	41	210	6.5	33	13.6	0
MMA-d_2	191.0	72.1	41	210	6.5	33	13.6	0
arginine	175.1	70.1	31	60	8.5	31	12.0	2
arginine-d_7	182.1	77.1	31	60	8.5	31	12.0	2

Table 2.2. Mass spectrometer settings for the analysis of arginine and methylated arginines.

2.4. The API Sciex 2000 Mass Spectrometer

The mass spectrometer used throughout the course of these studies was a Perkin-Elmer Sciex API 2000 triple quadrupole mass spectrometer, fitted with an electrospray ion source and coupled to a Perkin-Elmer HPLC autosampler and micropump (series 2000), this is shown schematically in figure 2.1.

Sample is injected by the autosampler into the mobile phase, pumped to the mass-spectrometer and sprayed into the electrospray ion source where, with the application of a high voltage, small charged droplets are formed. As the droplets evaporate (assisted by the application of heat) ions are ejected and are attracted by an applied voltage into the first quadrupole of the mass spectrometer (Q1), (figure 2.2.a). In Q1 the trajectories of the ions are manipulated by the further application of electric fields, and only ions of a selected charge to mass ratio (m/z) pass straight through to the second quadrupole (collision cell, Q2) (figure 2.2.b). In Q2 the ions collide with gas molecules (nitrogen) which, in addition to the application of electrical energy by the quadrupole, results in fragmentation of the ion (figure 2.2.c). The ion fragments pass to the third quadrupole where a particular fragment is selected according to its mass to charge ratio and is allowed to pass through to the detector. Software provided with the mass-spectrometer (Analyst-NT software) allows processing of the signal generated, and quantitation of results. The software provides facilities (Autotune facility) for the automatic determination of the energies, and mass spectrometer settings, required to produce the individual fragments.



HPLC system and autosampler. | Electrospray ion source/ nebuliser | Quadrupole one (Q1). Ion selection | Quadrupole two (Q2). Collision cell. | Quadrupole three (Q3). Ion selection | Detector

Figure 2.1. Schematic diagram of the API 2000 mass spectrometer. Sample is injected by the autosampler into the mobile phase and pumped to the mass-spectrometer. Small charged droplets are formed by the electrospray-ion-source/nebuliser. Ions of interest are selected according to their mass/charge ratio by the first quadrupole and pass to the second quadrupole where they are fragmented. Specific ion fragments are selected by the third quadrupole, again based on their mas to charge ratio, and are allowed to pass to the detector.

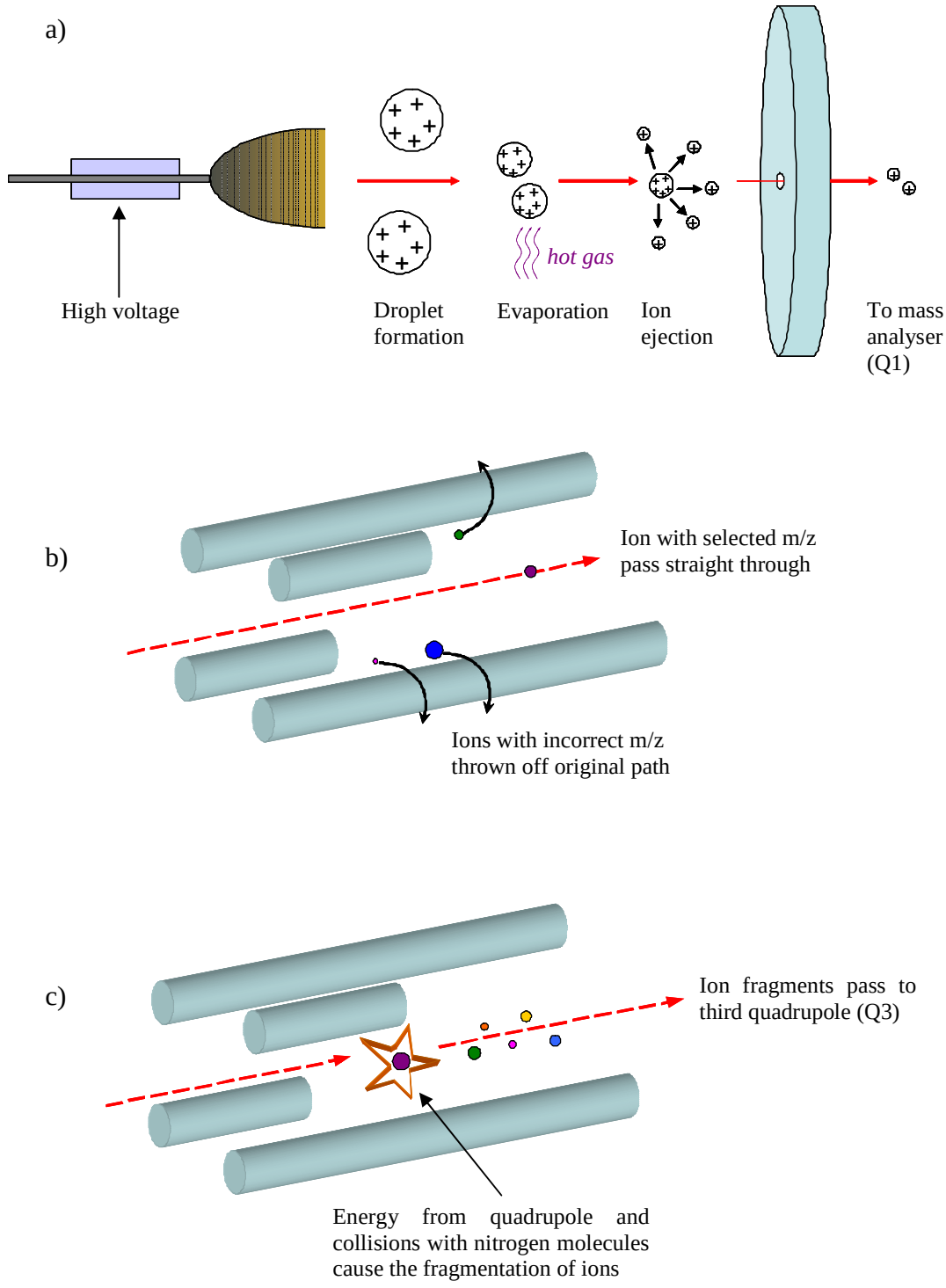


Figure 2.2. Ionisation, mass selection and fragmentation within the mass spectrometer. a) Injected sample is pumped to the electrospray ion source where if forms a spray of small charged droplets, which evaporate and eject ions which are then drawn by an electric field into the first quadrupole of the mass spectrometer. b) The trajectories of the ions are manipulated with electric fields and only those of the selected mass to charge ratio pass straight through. c) energy from the collision cell (quadrupole 2) and collisions with nitrogen molecules energise the ions, rendering them unstable and causing fragmentation.

3. Development of a Tandem-Mass Spectrometric Method for the Determination of Homocysteine, Cysteine and Methionine in Plasma and Urine.

3.1. Background

Plasma total homocysteine had been measured routinely in the Clinical Chemistry and Immunology Department at the Royal Sussex County Hospital since 1992, using an in-house HPLC method based upon that of Ubbink[71]. The method was laborious and time consuming, 20 samples taking more than two hours to prepare and approximately five hours for HPLC analysis, and as routine requests for homocysteine analysis continued to increase so an alternative method of analysis was sought. The department had recently taken possession of a bench-top tandem mass spectrometer (Applied Biosciences API Sciex 2000) and this, together with a publication describing the measurement of plasma total homocysteine by tandem mass spectrometry (Magera et al[92]), offered the possibility of developing a fast and simplified method for plasma homocysteine and related analytes.

3.2. Method Development and Optimisation

3.2.1. Determination of Fragmentation Patterns

The parent/daughter transitions for cysteine, homocysteine and methionine, along with the optimal potential settings for each, were determined by infusing a 10 μmol/L solution of each analyte at a flow rate of 5 μL per minute and using the AutoTune algorithm provided with the system software. The fragmentation patterns obtained for homocysteine, cysteine and methionine are shown in figures 3.1, 3.2, and 3.3.

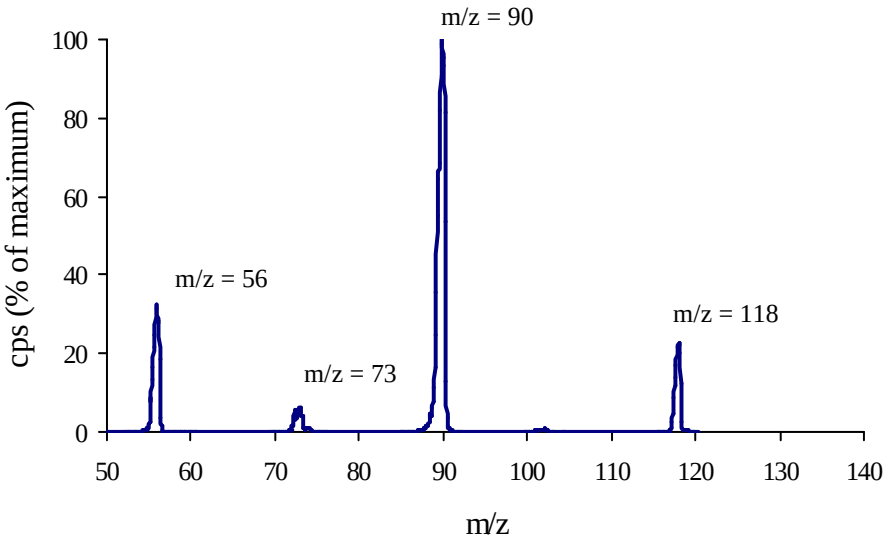


Figure 3.1. The mass spectrometric fragmentation pattern for homocysteine. This was determined by infusing a 10 µmol/L solution of homocysteine (containing 0.1% formic acid) into the mass spectrometer at a flow rate of 10 µL/min and scanning on the third quadropole. Homocysteine was produced by reduction of homocystine with DTT. The fragments were identified automatically by the mass spectrometer software.

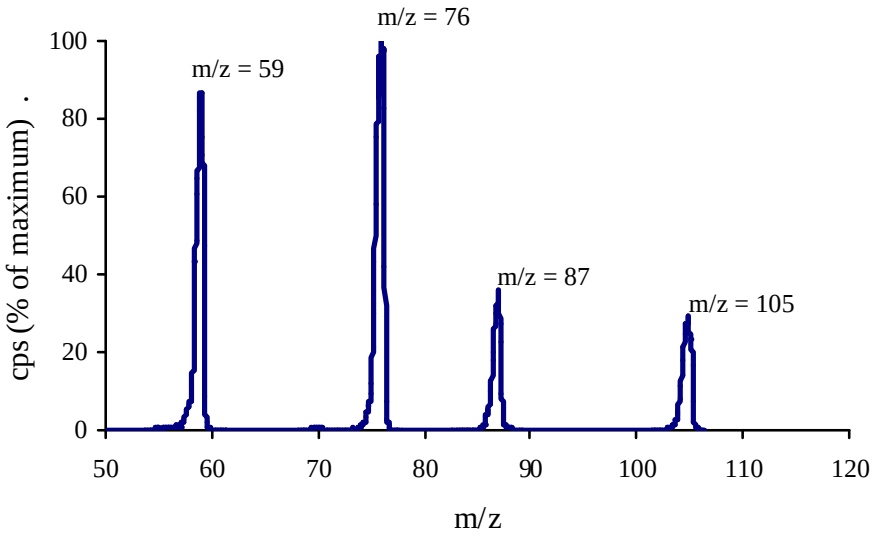


Figure 3.2. The mass spectrometric fragmentation pattern for cysteine. This was determined by infusing a 10 µmol/L solution of cysteine (containing 0.1% formic acid) into the mass spectrometer at a flow rate of 10 µL/min and scanning on the third quadropole. Cysteine was produced by reduction of cystine with DTT. The fragments were identified automatically by the mass spectrometer software.

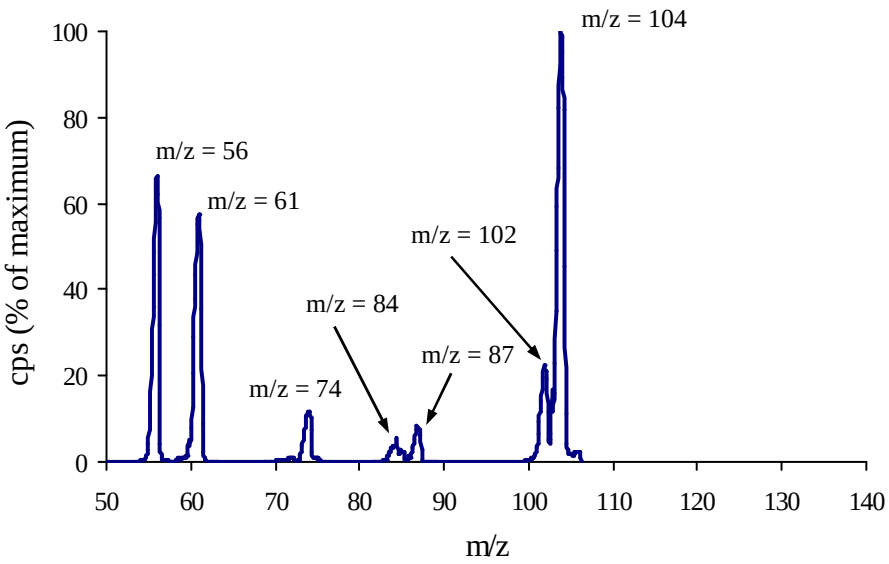


Figure 3.3. The mass spectrometric fragmentation pattern for methionine. This was determined by infusing a 10 μmol/L solution of methionine (containing 0.1% formic acid) into the mass spectrometer at a flow rate of 10 μL/min and scanning on the third quadropole. The fragments were identified automatically by the mass spectrometer software.

3.2.2. Nebuliser Optimisation.

Optimal nebulizer conditions were determined by repeat injection of 10 µL of a 10 µmol/L solution of homocysteine and stepwise variation of individual gas flows. The results obtained for the optimisation of the heater gas (gas source 2) are shown in figure 3.4 as an example of this process. The optimum conditions found were: curtain gas (air) 40 psi, ion source gas 1 (nitrogen) to 40 psi, ion source gas 2 (nitrogen) 60 psi and collision gas (nitrogen) 3 psi. The electrospray voltage was set to 5000 V.

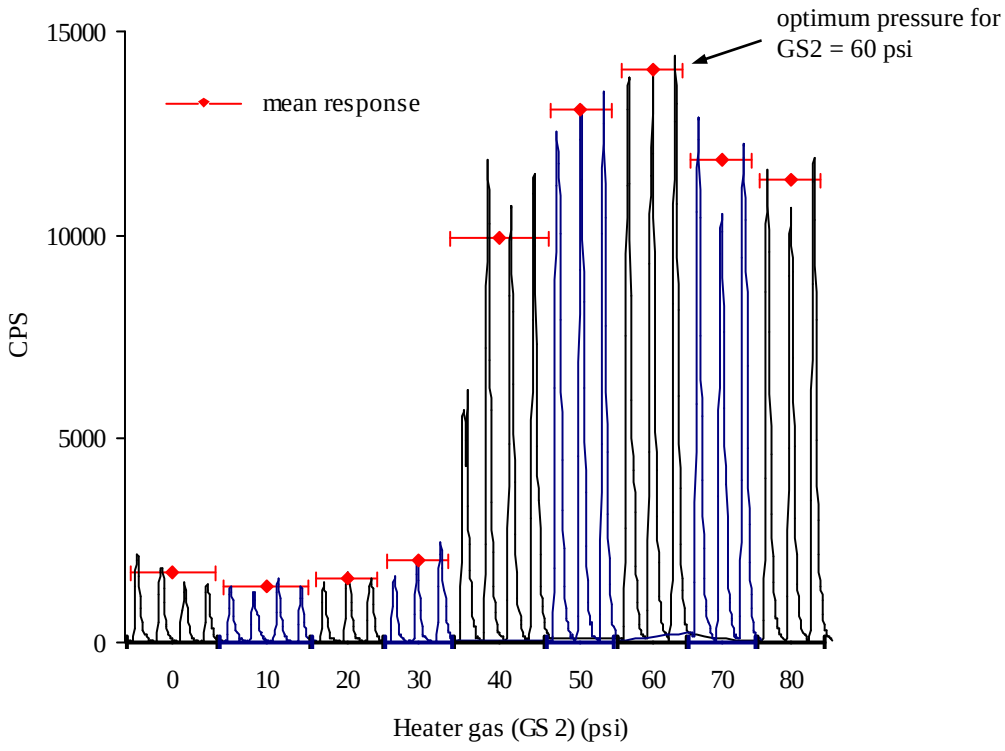


Figure 3.4. Optimisation of the heater gas. Ten microlitres of a 10 µmol/L solution of homocysteine was injected by the autosampler at 30 second intervals. The mass spectrometer was set to monitor the parent/daughter transition corresponding to homocysteine (m/z 136→90), and the pressure of the heater gas (GS2) was increased in 10 psi increments. The optimum gas pressure was determined as that which gave the greatest counts per second (height).

3.2.3. Temperature Optimisation and Effect on Suppression

The extent to which the analyte signal might be suppressed by other plasma constituents, and how this was affected by nebulizer temperature, was determined by monitoring the signal generated by an internal standard in both an aqueous preparation and a plasma preparation. Initially three solutions were prepared. Solution A consisted of 400 µL plasma and 200 µL reducing agent, solution B 400 µL internal standard and 200 µL reducing agent and solution C 400 µl deionised water and 200 µL reducing agent. After 15 minutes 2 mL of the protein precipitating agent was added to each, and after centrifugation equal parts of A and B were mixed for the plasma preparation and equal parts of B and C mixed for the aqueous preparation. The nebulizer temperature was set to 250 ^{O}C and the aqueous preparation injected five times, followed by five injections of the plasma preparation. The injection sequence was repeated with 50^{O}C increments in nebulizer temperature over the range 250-500 ^{O}C, allowing twenty minutes between each series for temperature equilibration (figure 3.5).

For cysteine-d_2, the mean signal obtained in the plasma preparation was 90% of that obtained for the aqueous preparation. For homocysteine-d_4 and methionine-d_3 it was 80%. There was no association between suppression and temperature (one-way ANOVA; cysteine $p = 0.167$, homocysteine $p = 0.455$, methionine $p = 0.657$). Increasing temperature produced an increase in absolute signal reaching a plateau at approximately 450^{O}C.

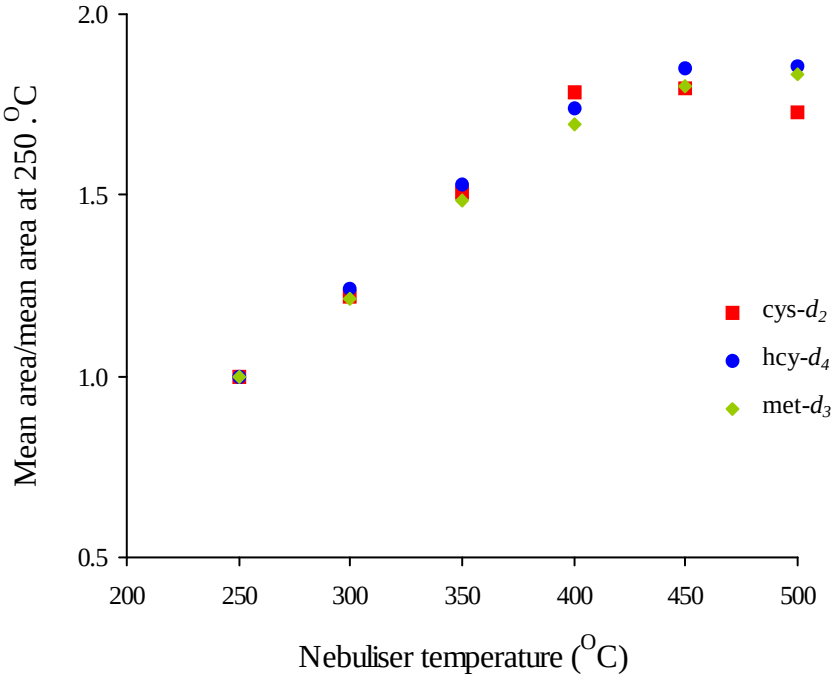


Figure 3.5. The effect of temperature on nebulisation and suppression. A solution of internal standard was prepared in water and plasma, and then each preparation was analysed by the mass spectrometer five times at each temperature, the latter being increased from 250^{O}C, in 50^{O}C increments, with twenty minutes being allowed between temperature changes for thermal equilibrium to be reached. For clarity only the results for the aqueous preparation are shown.

3.3. Method Validation

3.3.1. Linearity Studies

Preliminary experiments to assess the linearity of the method were carried out using aqueous standards, the concentration of the highest standard (following reduction) being 2000 µmol/L cysteine, 200 µmol/L homocysteine and 2000 µmol/L for methionine.

Although the standard curves for homocysteine and methionine were linear for the concentration range examined a distinctly curved response was obtained for cysteine. The

cause of this alinearity was determined as being due to isobaric interference of the internal standard, cysteine-d_2, by cysteine. Of the cystine isotopes available, cystine-d_4, which on reduction yields cysteine-d_2, had been chosen for use as one of the internal standards, partly on considerations of cost. A close examination of the molecular weight distribution for cysteine, taking into account the relative abundances of the naturally occurring isotopes, showed that whilst the predominant form of cysteine has a molecular weight of 121 approximately 5% of naturally occurring cysteine has a molecular weight of 123, the same as the predominant form of the labelled internal standard. The relatively high proportion of cysteine with a molecular weight of 123 is due to the fact that sulphur naturally exists as two isotopes, ${}_{16}S^{32}$ and ${}_{16}S^{34}$, with relative abundances of 95.0% and 4.2% respectively. Cysteine, and other substances containing one sulphur atom, therefore also exists mainly in two molecular weight forms in approximately the same proportions. This does not in itself cause problems with quantitation since standards and samples will both contain the same isotopic forms in the same relative proportions. Problems do arise though with internal standardisation as approximately five percent of cysteine has a molecular weight of 123, the same as the major form of cysteine-d_2 and both of these ionise and fragment in the mass spectrometer to produce a daughter ion of the same mass, the minor cysteine component therefore produces an isobaric interference with the major cysteine-d_2 component. This difficulty was overcome by using the higher molecular weight form of cysteine-d_2 for internal standardisation.

Further linearity studies were performed using aqueous, plasma and urine based standards. The plasma and urine standards were prepared by first producing a stock standard containing 20 mmol/L cystine (equivalent to 40 mmol/L cysteine after reduction), 2 mmol/L homocystine (equivalent to 4 mmol/L homocysteine after reduction) and 40 mmol/L methionine in 0.1 mol/L HCl. Standards were prepared in HCl as cystine is poorly soluble in water. Intermediate standards were prepared by further dilution of stock standard in 0.1 mol/L HCl. Finally working standards were prepared by addition of intermediate standard (50 µL) to either pooled plasma (950 µL) or pooled urine (950 µL). All standards were analysed in triplicate, in random order, within one batch. The linearity of the method was determined as being to at least 2000 µmol/L for cysteine, 200 µmol/L

for homocysteine and 2000 µmol/L for methionine for both plasma and urine. A chromatogram for a typical plasma sample is shown in 3.6 and the standard curves obtained for the aqueous, plasma and urine based standards are shown in figures 3.7, 3.8, and 3.9.

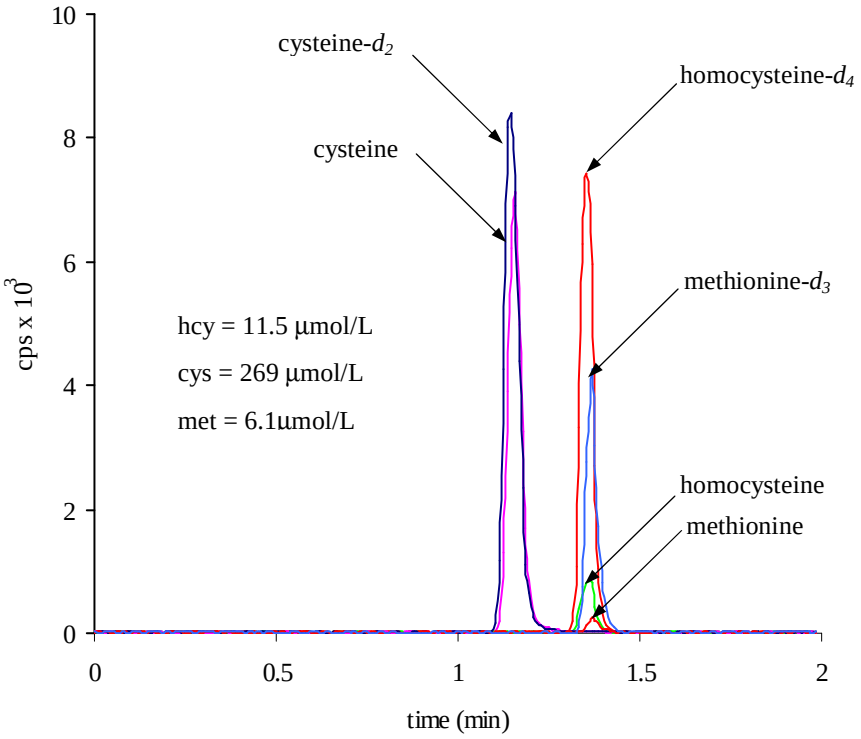


Figure 3.6. An example of a typical chromatogram of a plasma sample, analysed as described on p43.

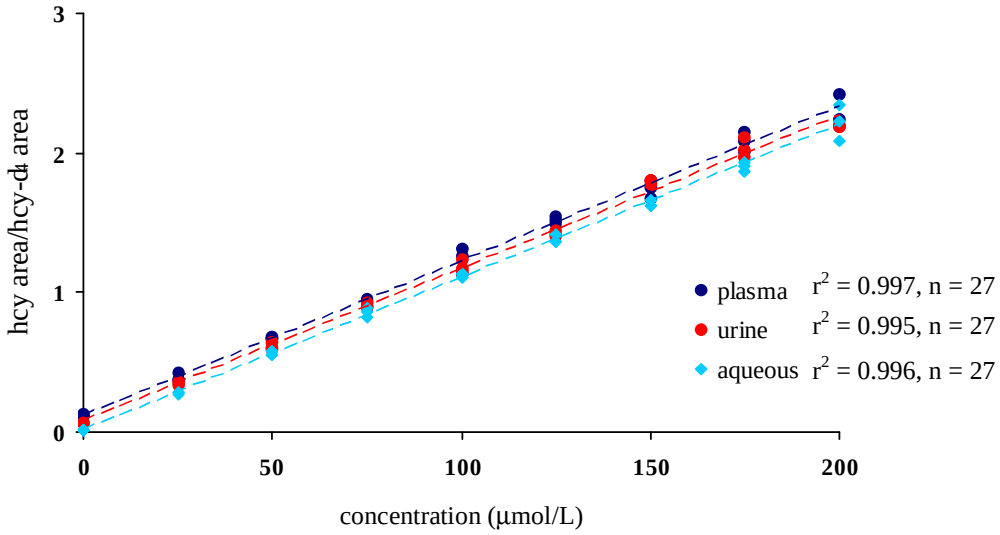


Figure 3.7. Standard curves for homocysteine. For plasma and urine standards, pooled plasma or urine was spiked with an aqueous standard (19 parts plasma or urine:1 part standard). All standards were analysed in triplicate, in random order. The results for each standard are plotted individually, but many points are coincidental.

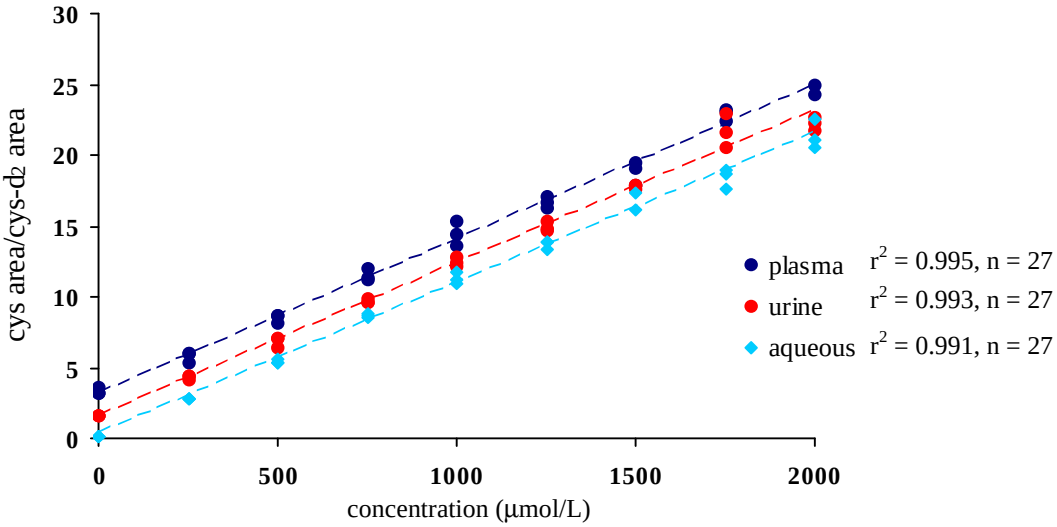


Figure 3.8. Standard curves for cysteine. For plasma and urine standards, pooled plasma or urine was spiked with an aqueous standard (19 parts plasma or urine:1 part standard). All standards were analysed in triplicate, in random order. The results for each standard are plotted individually, but many points are coincidental.

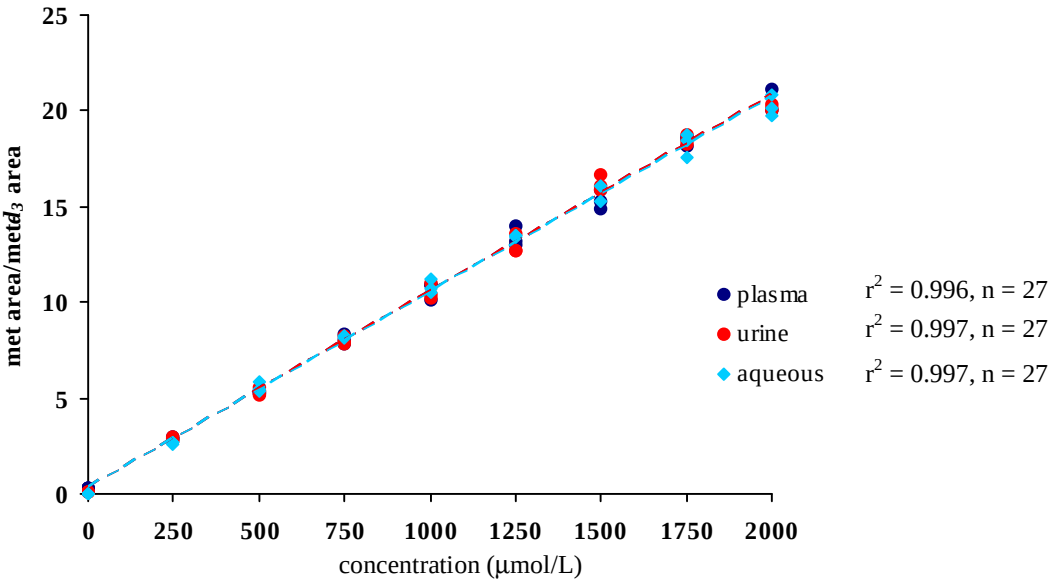


Figure 3.9. Standard curves for methionine. For plasma and urine standards, pooled plasma or urine was spiked with an aqueous standard (19 parts plasma or urine:1 part standard). All standards were analysed in triplicate, in random order. The results for each standard are plotted individually, but many points are coincidental.

3.3.2. Precision

Within-batch and between-batch precision was determined at three concentrations using pooled human plasma or pooled human urine (with stock standard added to the plasma or urine pool for the intermediate and high concentrations). Twenty replicates were analysed in each case. Results for within batch-precision and between batch-precision are shown in table 3.1.

Sample type	Analyte	Within-batch		Between-batch	
		Mean (μmol/L)	%CV	Mean (μmol/L)	%CV
Plasma	homocysteine	10.1	3.5	10.7	9.1
		33.5	3.3	32.1	5.8
		63.4	3.1	62.6	5.6
	cysteine	310	4.9	323	10.5
		538	3.9	653	14.1
		835	4.0	1117	14.4
	methionine	30.6	2.9	30.4	11.1
		50.0	3.7	49.5	11.8
		80.2	3.2	83.0	12.7
Urine	homocysteine	11.5	4.5	10.8	7.5
		63.0	2.5	59.7	5.3
		108.9	2.5	104.5	5.2
	cysteine	319	4.2	313	7.7
		832	3.8	802	3.0
		1296	4.1	1260	7.0
	methionine	6.5	5.2	6.7	14.8
		60.0	3.4	54.5	4.6
		107.7	3.1	99.6	5.8

Table 3.1. Within- and between-batch precision for plasma and urine total homocysteine, total cysteine and methionine. Pooled plasma or urine was used for the low value samples. For intermediate and high values, pooled plasma or urine was spiked with an aqueous standard (1 part standard to 19 parts plasma or urine). Twenty replicates were analysed at each concentration.

3.3.3. Recovery

Samples for recovery studies were prepared by adding stock standard (at two different concentrations) or 0.1 mol/L HCl to twenty different plasma and twenty different urine samples (20 μL standard to 180 μL plasma, 50 μL standard to 950 μL urine). Each preparation was analysed in duplicate. The effects of using HCl as diluent rather than water was examined by using ten different plasma samples, splitting each into two portions, and adding 0.1 mol/L HCl to one portion and deionised water to the other (50 μL 0.1 mol/L HCl or water, 950 μL plasma). No statistically significant difference was observed in adding 0.1 mol/L HCl to samples as compared with deionised water (Student's paired

t-test: p = 0.73 for cysteine, p = 0.48 for homocysteine and p = 0.15 for methionine). The results of the recovery experiment are shown in table 3.2. The use of aqueous standards led to a lower mean recovery compared to using matrix-matched standards (by approximately 5% for all analytes) and so for comparison studies and the routine analysis of samples matrix-matched standards were used.

Sample type	Analyte	Added concentration (μmol/L)	Aqueous standards		Matrix matched standards	
			Mean recovery (%)	%CV	Mean recovery (%)	%CV
Plasma	homocysteine	20	93.1	4.4	100.9	4.3
		50	93.9	3.2	101.8	3.2
	cysteine	200	94.6	10.1	97.0	10.1
		500	97.6	6.1	100.1	6.0
	methionine	20	94.7	8.6	102.7	8.6
		50	93.4	4.4	101.1	4.4
Urine	homocysteine	20	94.2	4.1	97.5	4.1
		50	90.6	4.9	93.7	5.0
	cysteine	200	92.5	9.5	103.7	9.5
		500	89.7	7.6	100.5	7.5
	methionine	20	92.5	3.8	97.3	3.7
		50	89.5	5.4	94.2	5.4

Table 3.2. Recovery of homocysteine, cysteine and methionine from plasma and urine. Baseline samples were prepared by the addition of 0.1 mol/L HCl, spikes by the addition of standard in 0.1 mol/L HCl (1 part HCl or standard to 19 parts sample). Homocystine and cystine were used for preparation of standards, with homocysteine and cysteine being subsequently generated by reduction of samples with DTT. Twenty samples were prepared at each concentration and were analysed in duplicate. The results were calculated using both aqueous and matrix matched standards.

In a review of tandem mass spectrometric methods for the analysis of homocysteine[58] the above results for recovery and precision compared well with other similar methods (table 3.3).

Reference, year of publication	Mean recovery (%)	Mean between batch precision, %cv (number)	Mean within batch precision, %cv (number)
Hempen et al[102], 2008	97.5 (4)	2.3 (20)	5.0 (12)
Li et al, 2008[196]	98.6	3.2	4.0
Huang et al[99], 2007	102	4.9 (45)	not given
Rafi et al[100], 2007	101.1 (12)	4.5 (9)	5.8 (27)
Weaving et al[197], 2006	101.4 (20)	3.3 (60)	6.8 (60)
Kuhn et al[98], 2005	94.7	not given	not given
Tuschl et al[97], 2005	79.8	10.2 (10)	11.5 (5)
Nelson et al[96], 2005	not given	2.1 (2)	6.0 (6)
Nelson et al[94], 2003	not given	3.6 (60)	not given
McCann et al[95], 2003	not given	6.0 (10)	10.3 (10)
Magera et al[92], 1999	96.0	4.3 (18)	4.1 (18)

Table 3.3. Precision and recovery data for published methods for the analysis of plasma homocysteine by tandem mass spectrometry. Adapted from Rafii et al[58].

3.3.4. Limits of Detection and Quantitation

The limit of detection and limit of quantitation were determined by analysing ten sample blanks (water in place of sample). The signal (baseline) obtained from each analysis was integrated using Analyst-NT software to obtain a measure of noise. The limit of detection was then calculated as 3 x blank sd and the limit of quantitation as 10 x blank sd. The limits of detection for homocysteine, cysteine and methionine were 0.2 μmol/L, 0.5 μmol/L and 0.2 μmol/L respectively, while the limits of quantitation were 0.4 μmol/L, 0.9 μmol/L and 0.4 μmol/L respectively.

3.3.5. Homocysteine method comparison.

Comparison studies for plasma total homocysteine were performed on two different types of analyser. The Drew Scientific Ltd DS 30 is an enclosed automated HPLC system (Drew Scientific Ltd, Barrow-in-Furness, UK) using pre-column derivatisation with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF) and fluorescence detection. This analyser was chosen because it gave comparable results to our previously used in-house HPLC system. The IMx analyser (Abbott Diagnostics, Maidenhead, UK) uses an enzymic reaction to first convert homocysteine to adenosyl homocysteine which is then measured using fluorescence polarisation immunoassay. Both the commercial systems were calibrated and used to assay plasma samples according to the manufacturer's instructions. The results of the comparisons are shown in figures 3.10. and 3.11.

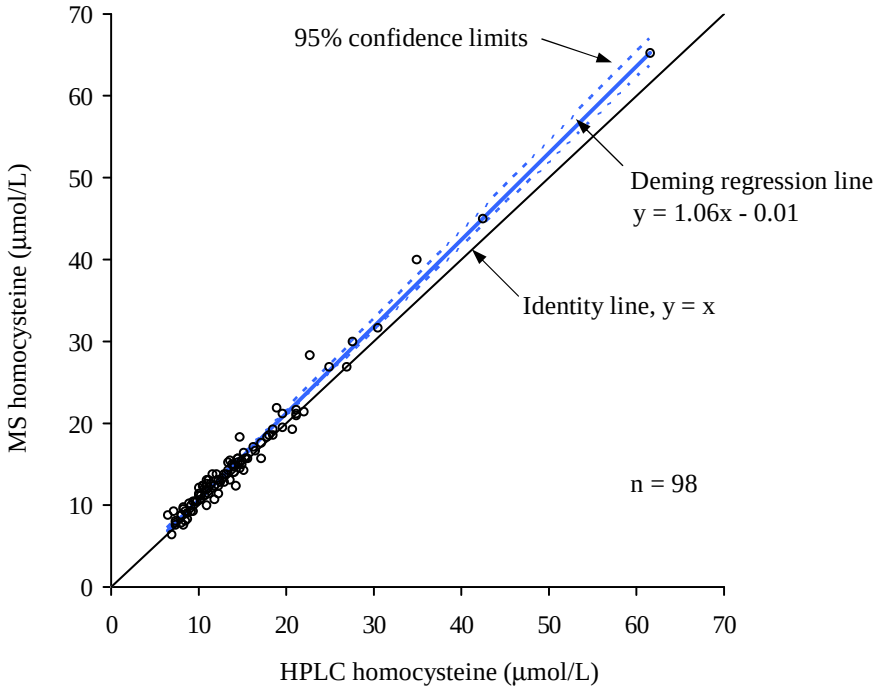


Figure 3.10. Comparison of plasma total homocysteine results obtained by mass spectrometry and by HPLC (Drew 30).

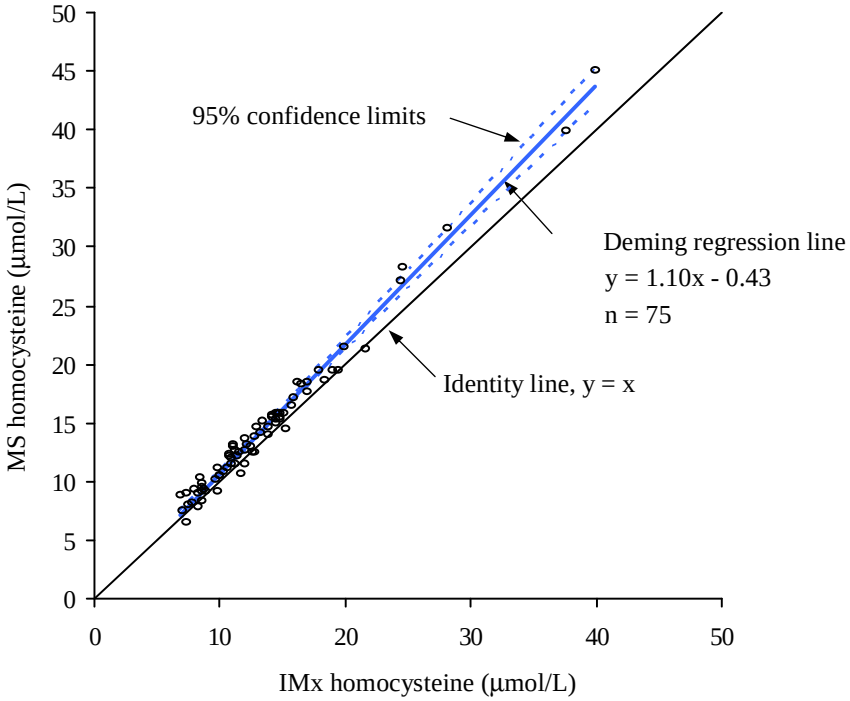


Figure 3.11. Comparison of plasma total homocysteine results obtained by mass spectrometry and by FPIA (Abbot IMx).

Plasma total homocysteine measured by tandem mass spectrometry produced results higher than those produced by either the Drew analyser or the Abbot IMx analyser. The respective Deming regression equations were: MS = 1.06 HPLC - 0.01, and MS = 1.10 IMx - 0.43. Others have also reported discrepant results between methods and inspection of external quality assessment scheme reports for homocysteine shows that between-laboratory comparability is problematic.

The inter-method differences may be due in part to the use of different calibration materials and procedures. The LC-MS/MS method employs matrix-matched homocystine standards whilst the Drew DS 30 uses an aqueous homocysteine calibrant.

For the IMx method calibration is not performed using homocysteine but adenosylhomocysteine is used instead. Plasma samples are initially treated enzymically to

convert homocysteine to adenosylhomocysteine, but this step is not required for the calibrants. Not only are there issues regarding the material used for calibration but samples and standards are not subject to identical steps.

Although the comparison studies were only performed using matrix matched standards for the MS assay the possible effects of having used aqueous standards may be modelled using the results of the recovery experiments detailed above. In these experiments the mean recovery of homocysteine added to plasma was 93.5% when aqueous standards were used for calibration and 101.4% when matrix matched standards were used. If these values are representative of the general behaviour of plasma samples with regard to the use of different calibration procedures in the MS assay, then they may be used to 'recalculate' the results obtained to give values that would be expected had aqueous standardisation been used. When this is performed there is much closer agreement between the MS method and both the Drew 30 and IMx methods, the Deming regression equations becoming: MS = 0.98 HPLC + 0.01, and MS = 1.02 IMx - 0.39 (figures 3.12 and 3.13).

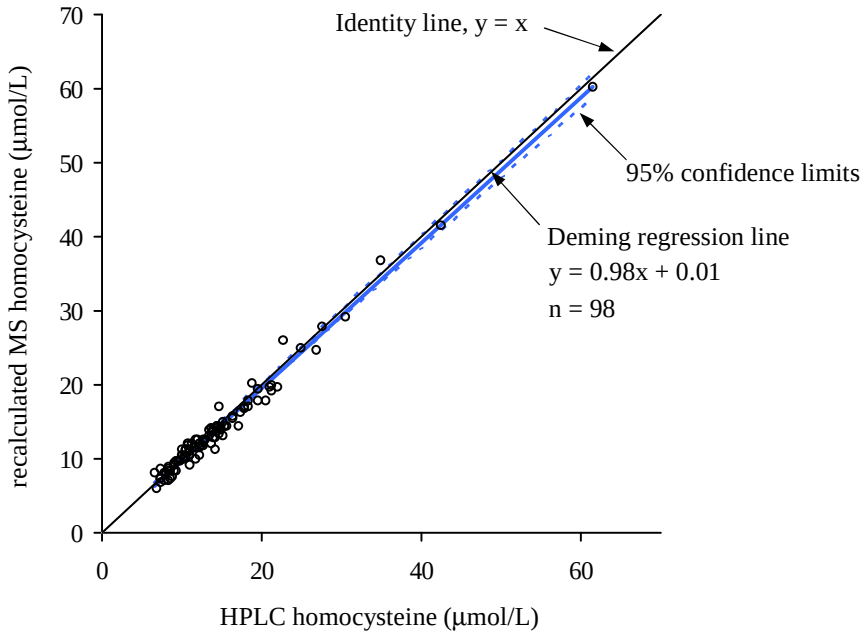


Figure 3.12. Comparison of plasma total homocysteine results obtained by mass spectrometry to those obtained using HPLC (Drew 30), after adjusting for calibration effects. The results obtained by mass spectrometric analysis were recalculated, using results obtained from the recovery experiments, to simulate the effect of using aqueous standards instead of plasma based standards.

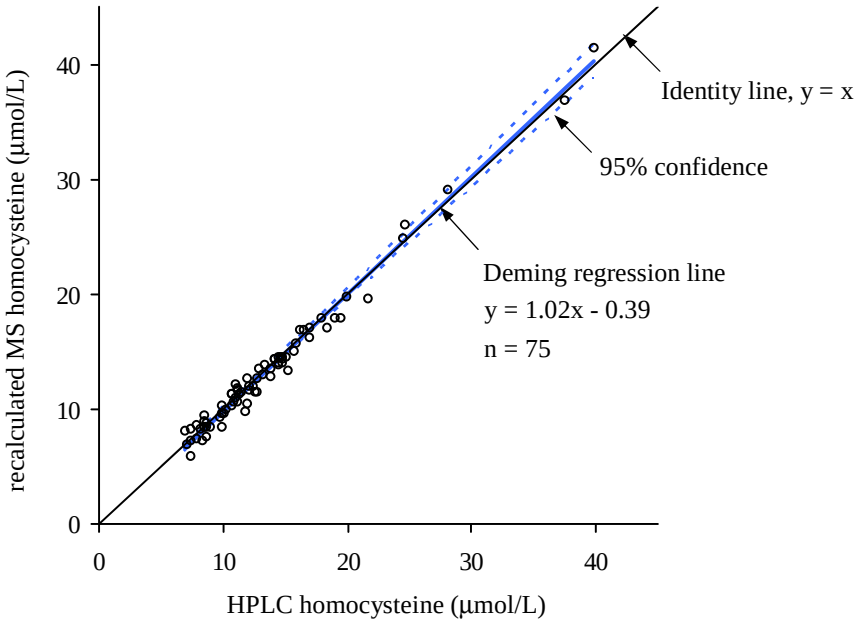


Figure 3.13. Comparison of plasma total homocysteine results obtained by mass spectrometry to those obtained using FPIA (Abbot IMx), after adjusting for calibration effects. The results obtained by mass spectrometric analysis were recalculated, using results obtained from the recovery experiments, to simulate the effect of using aqueous standards instead of plasma based standards.

At the moment, a universally agreed reference material for the calibration of plasma homocysteine assays is not available

3.3.6. Methionine method comparison.

For comparison of plasma methionine, samples were analysed by an in-house HPLC based amino acid screening system. As all the samples available for analysis had methionine concentrations falling within a narrow range, the methionine concentration of some samples was increased by 50, 100, 150 or 200 µmol/L (five of each) by the addition of 50 µL standard to 950 µL sample. Methionine measurements by tandem mass-spectrometry was approximately 30% higher than measurements by HPLC, the Deming regression equation for the comparison being MS = 1.34 HPLC - 5.85. The results of the comparison are shown in figure 3.14.

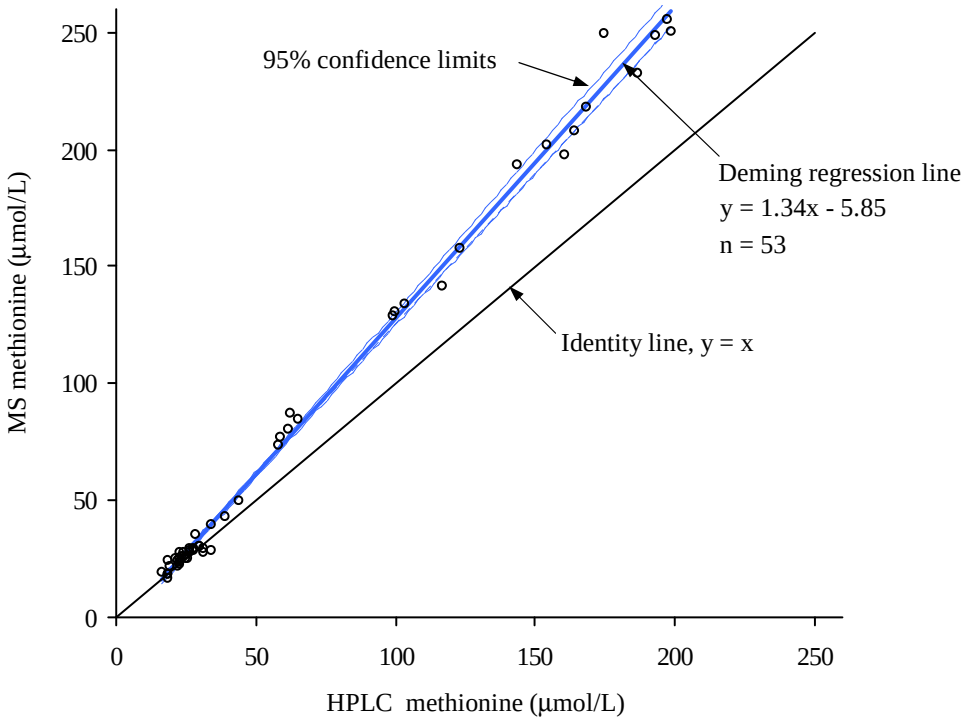


Figure 3.14. Comparison of plasma methionine results obtained by mass spectrometry and by an in-house HPLC method[193].

The HPLC method serves as a first-line screening test to detect abnormal amino acid patterns in sick neonates and not as a quantitative amino acid analyser. With this method, plasma proteins are first precipitated with ethanol and then the amino acids are derivatised with phenylisothiocyanate; nor-leucine is used as internal standard, which may behave very differently from methionine with regard to precipitation and derivatisation. The method is calibrated with an aqueous standard which may also behave differently to plasma samples. No recovery data was available for this method. A series of aqueous and plasma standards analysed by HPLC gave calibration curves with gradients in the ratio 1:1.43 (plasma:aqueous). Therefore, it is likely that the HPLC method shows poor recovery for plasma methionine, and this could account for the difference in the measurements obtained by mass spectrometry.

3.3.7. Cysteine method comparison.

Urine cysteine comparisons were carried out using an in-house colorimetric assay based on the cyanide-nitroprusside reaction. Results are shown in figure 3.15. Urine cysteine measurements obtained by mass spectrometry were consistently lower than those obtained by the colorimetric assay, the Deming regression equation being: MS = 0.86 colorimetric - 69.3.

Although useful for detecting and monitoring cystinuria, the cyanide-nitroprusside reaction is known to suffer from a number of interferences and is probably best regarded as a semiquantitative assay, and this may account for the lack of agreement between the two methods. Plasma cysteine was also measured by LC-MS/MS, but the results were not subjected to a comparative procedure since no routine method was available.

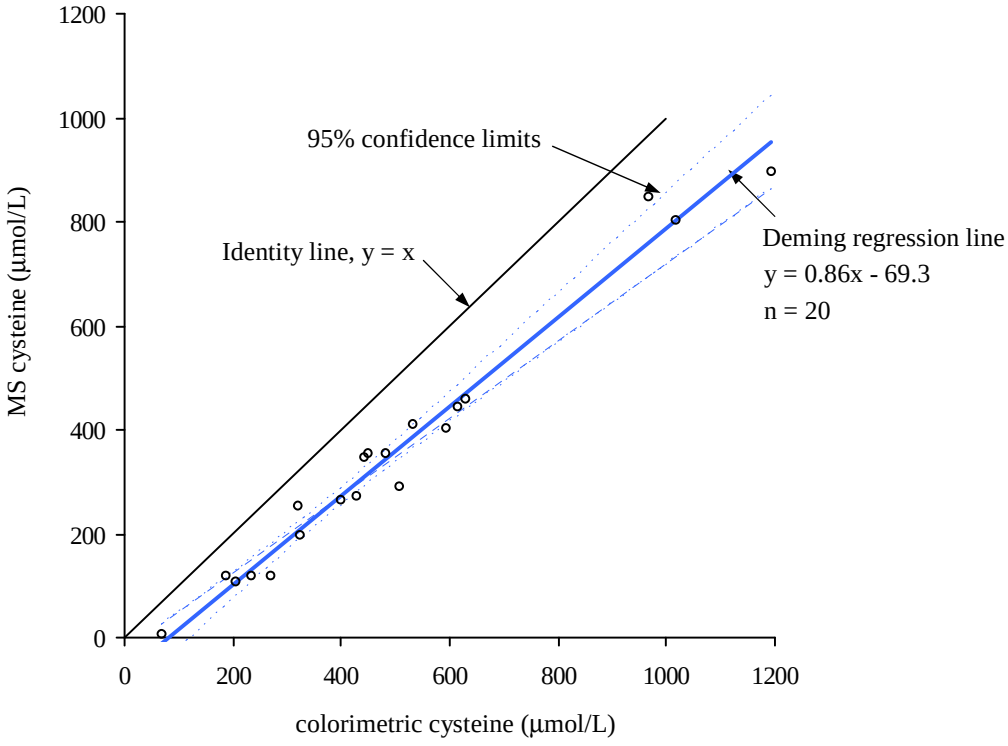


Figure 3.15. Comparison of urine cysteine results obtained by mass spectrometry and by an in-house colorimetric assay.

3.4. Conclusion

A method for the simultaneous measurement of total homocysteine, total cysteine and methionine in plasma and urine has been described. Results obtained by mass spectrometry for plasma homocysteine compare with accredited commercially available kits (Drew DS 30 HPLC and Abbott IMx immunoassay). The technique requires only 50 μL of plasma, is relatively fast and uses less expensive reagents than the commercial systems. The simultaneous assay of methionine helps distinguish cystathionine beta synthase deficiency from other causes of hyperhomocysteinaemia and the replacement of a routine assay for the determination of urinary cystine that utilizes sodium cyanide with a more specific and less hazardous method is an additional benefit.

4. Development of a Tandem-Mass Spectrometric Method for the Determination of Arginine and Methylated Arginines in Plasma and Urine.

4.1. Method Development

4.1.1. Initial Considerations

The aim was to develop a method that would ideally measure ADMA, SDMA, MMA and arginine simultaneously and which was sufficiently accurate and precise to allow for the reliable interpretation of the results in clinical practice. Consideration was also given to the routine working practises of the department in which the method was to be used. A method using an isocratic mobile phase would be more convenient over a method requiring gradient separation, as one of the two solvent lines of the HPLC system attached to the mass spectrometer was in regular use for other analyses, short run times would enable a greater sample throughput and the preparation of samples should be as quick, simple and robust as possible so that the method could be performed by a variety of staff with differing levels of skill. Methods for the analysis of dimethylarginines have been reviewed in section 1.3.5 and many of these have made use of OPA derivatisation to facilitate the chromatographic separation of ADMA and SDMA but have disadvantages that include long run times and the need for gradient elution, nevertheless derivatisation by OPA was thought to be a useful starting point for the development of a tandem mass spectrometric method.

4.1.2. The Use of O-phthaldialdehyde

The fragmentation patterns of OPA-ADMA and OPA-SDMA were obtained by derivatising 1 mmol/L solutions of ADMA and SDMA (separately), as described in section 2.3 (figure 4.1). The derivatised amino acids were diluted 1/10 in 0.1% formic acid and then infused into the mass spectrometer at 5 μL/min. The infused solutions were initially scanned using only the first quadrupole of the mass spectrometer (Q1 scan) over the mass range 300 – 400 amu to ensure that the predicted product, m/z = 379, had indeed been

formed. This peak was observed in the Q1 scan but two others, with m/z = 319 and m/z = 334, were also seen. In comparing the Q1 scans obtained for OPA-ADMA and OPA-SDMA to that obtained from infusing a blank solution consisting of OPA reagent diluted in 0.1% formic acid, it was apparent that these additional peaks were not associated with contaminants in the reagents but were additional products being formed by the derivatisation of dimethylated arginines. The Autotune software facility (section 2.4.) was used for determining the fragmentation of each of the products. On examining the fragmentation patterns obtained it was noted that there appeared to be the production of three parent/daughter combination which were unique to ADMA (m/z: 378.9 → 230.1, 378.9 → 257.2, and 378.9 → 290.2) and also two parent/daughter combinations which were unique to SDMA (m/z 378.9 → 205.2 and 378.9 → 244.2) . There were no unique daughter ions observed for the parent ions with m/z 319 and 334.

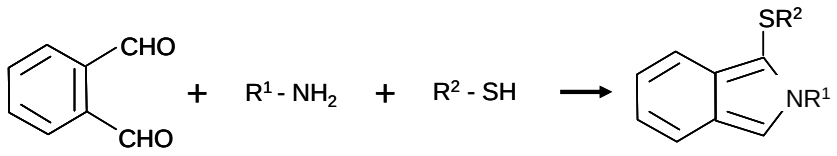


Figure 4.1. The reaction of amino acids with o-phthaldialdehyde and a conjugating thiol.

4.1.3. Stability problems with OPA.

Whilst carrying out the determination of the fragmentation patterns for the different OPA-DMA derivatives it was observed that the relative proportions of the parent ions, for both ADMA and SDMA derivatives, changed with time. In particular the product with m/z 379 decreased whilst the product with m/z 319 increased. The decrease in intensity of the first product was not surprising as OPA-amino acid derivatives are well known for being unstable[198]

To examine the rate at which the concentrations of the various products changed, and whether this might be affected by the presence of the formic acid which had been added to

promote ionisation, a 10 mmol/L ADMA standard was derivatised using OPA/mercaptoethanol as reagent (10 μL standard + 90 μL reagent). To this was then added either 900 μL 0.1% formic acid or 900 μl deionised water. The preparation was then quickly transferred to the mass spectrometer for analysis. Twenty microlitres of the preparation was injected into the mass spectrometer, using 0.1% formic acid as the carrier solution, pumped at 50 μL per minute. Scanning over the mass range 40 – 400 amu was performed using the first quadrupole of the mass spectrometer. Each scan cycle took 20 s and was repeated continuously for 5 minutes, at which time the sample was re-injected and the scanning process repeated. A total of 12 consecutive injections for each preparation was performed. A nebuliser temperature of 500 ^{o}C was used, the other parameters being set to the equipment's default conditions The data was analysed by extracting the total ion count for each mass from the sum of all the scans performed within one 5 minute injection cycle. In previous experiments the products which had formed from the reaction of OPA with either ADMA or with SDMA all had m/z greater than 300 amu. The reactants had not been examined below 300 amu to determine whether there were further products/breakdown products being formed with lower molecular weights, and therefore to determine whether this might be so, an extended scanning range, 40 – 400 amu, was chosen for this experiment. No peaks corresponding to product formation were seen other than those previously noted with m/z = 319, 334 and 379. The change of concentration of each product with time is figure 4.2.

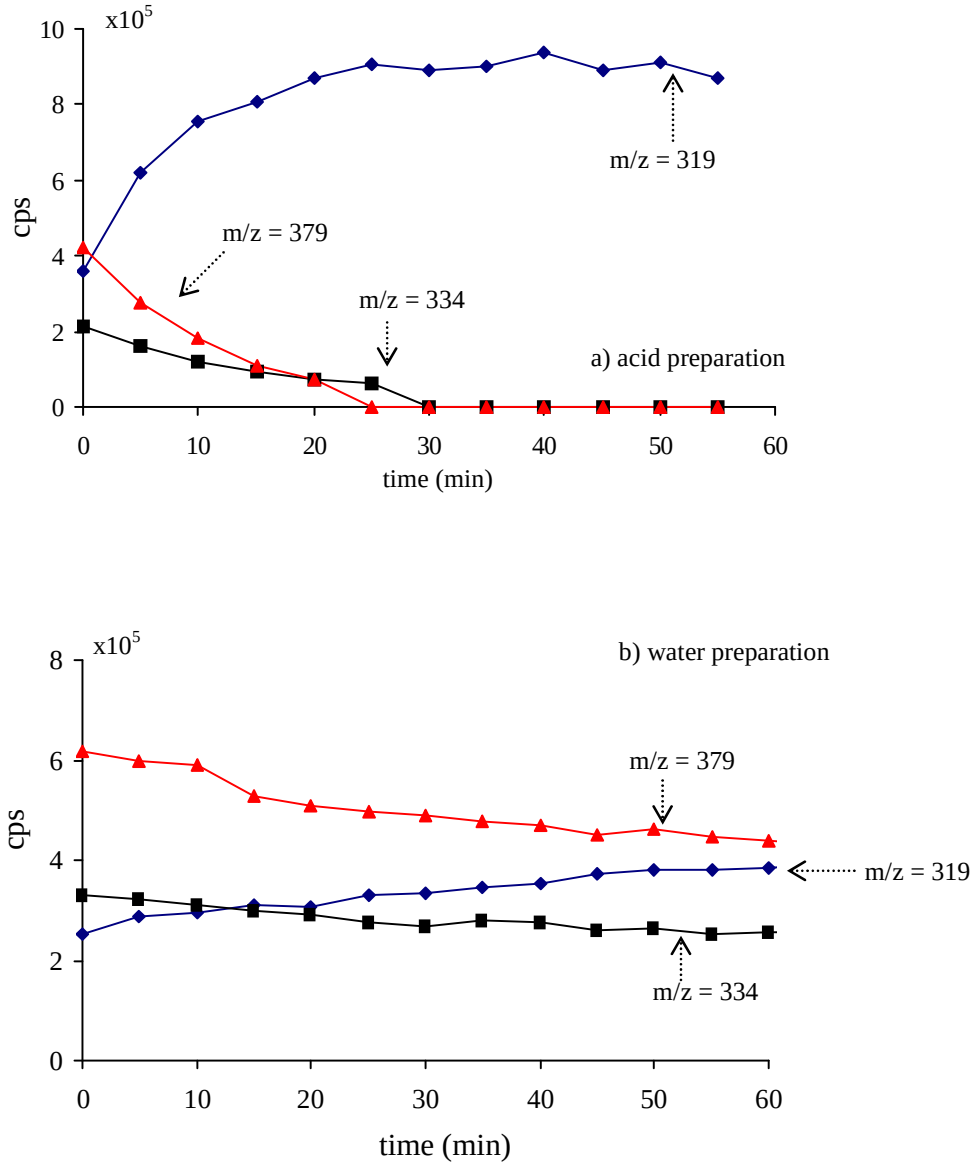


Figure 4.2. Changes in ADMA-OPA products with time. A 10 mmol/L ADMA standard was derivatised with OPA/mercaptoethanol reagent (10 µL standard + 90 µL reagent) and 900 µL of 0.1% formic acid was then added (a). Twenty microlitres of the preparation was injected into, and analysed by, the mass spectrometer with repeat injections every five minutes for one hour. The experiment was then repeated, but the derivatised standard was diluted with deionised water instead of formic acid (b).

The ADMA standard used for this experiment was highly concentrated (10 mmol/L). The way in which it was derivatised and diluted resulted in a preparation with an OPA-ADMA concentration of 100 μmol/L, assuming that all the ADMA had indeed derivatised. The latter was a reasonable assumption in that the OPA was in large excess by comparison to ADMA. Although this concentration was much higher than that expected to be found in plasma samples (approximately 1 μmol/L) its use was required due to the fact that in a preliminary experiment, using a standard with an ADMA concentration of 1 mmol/L (resulting in a final concentration of 10 μmol/L for OPA-ADMA) the peaks observed had very low intensity.

For both OPA-ADMA diluted in formic acid and also diluted in water the products with m/z = 379 and m/z = 334 decreased with time whilst the product with m/z = 319 increased. These changes were most rapid in the acid preparation, with the peak at 379 being virtually undetectable by 25 minutes. Although the changes were slower when the product was diluted with water instead of acid, at 60 minutes the peak at 379 had decreased by 30% of its initial intensity and this was considered to be extremely undesirable for developing a method for routine use in which even a moderately sized batch of samples (along with standards and controls) could take several hours for analysis. The instability of the products would not have been a problem had the mass spectrometer autosampler been able to mix sample with reagent just prior to injection, but this facility was not available on the system used.

4.1.4. Other derivatising reagents and procedures

Given that derivatisation with OPA led to the formation of products which could be identified by unique daughter ions, the possibility existed that the use of other means of derivatisation might also form products with unique daughter ions, but hopefully with increased stability. Derivatisation with OPA utilising a variety of different conjugating agents was examined and also derivatisation with naphthalene-2,3-dicarboxaldehyde (NDA) (figure 4.3) and phenylisothiocyanate (PITC) (figure (4.4).

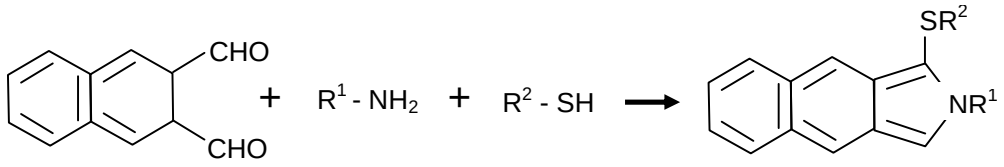


Figure 4.3. The reaction of amino acids with naphthalene-2,3-dicarboxaldehyde (NDA).

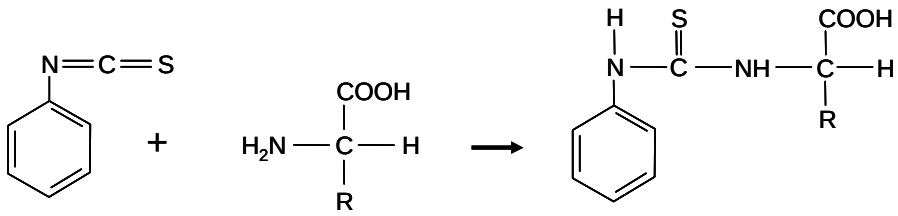


Figure 4.4. The reaction of amino acids with phenylisothiocyanate (PITC).

Until this point it had been assumed that ADMA and SDMA, without derivatisation, would not form unique daughter ions, since this had not been reported in the literature and published methods focussed upon chromatographic separation of the two isomers. As part of the work with differing methods of derivatisation, the fragmentation of ADMA and SDMA without derivatisation was also examined to test the validity of this assumption

The majority of this work was carried out looking for the production of daughter ions with a mass greater than 55 (the default setting for the API 2000), the data in the literature suggested that useful fragments with a lower mass might also be formed[199] and so an extended mass range was used when examining PITC derivatives, and the fragmentation of ADMA and SDMA without derivatisation.

The findings of these experiments are shown in table 4.1. Unique daughter ions were observed for both ADMA and SDMA without derivatisation (figure 4.5), and also following derivatisation with PITC; no other derivatisation procedure resulted in the generation of unique daughter ions for both ADMA and SDMA.

Derivatising Reagent	Parent ion (m/z)	Daughter ion (m/z)	Observations	Quantity of fragment (% of most abundant fragment)
OPA-mercaptoethanol [a)]	378.9	230.1	unique for ADMA	12
	378.9	257.2	unique for ADMA	8
	378.9	290.2	unique for ADMA	9
	378.9	205.2	unique for SDMA	15
	378.9	244.2	unique for SDMA	15
OPA-mercaptoethanol [a)]	335.0	290.2	unique for ADMA	9
	335.0	105.0	unique for SDMA	6
	335.0	205.1	unique for SDMA	15
	335.0	244.0	unique for SDMA	14
OPA-mercaptoethanol [a)]	319.0	119.1	unique for ADMA	9
	319.0		no unique daughter for SDMA	
OPA-mercaptopropanol [a)]	393.0	142.2	unique for ADMA	7
	393.0		no unique daughter for SDMA	
OPA-mercaptopropanol [a)]	349.0	257.2	unique for ADMA	28
	349.0	111.0	unique for SDMA	13
OPA-mercaptoundecanol [a)]	505.1		no unique daughter ions	
OPA-mercaptoundecanol [a)]	461.9	97.2	unique for ADMA	8
	461.9	203.1	unique for ADMA	22
	461.9		no unique daughter for SDMA	
OPA-monothioglycerol [a)]	408.9	258.3	unique for ADMA	12
	408.9	205.1	unique for SDMA	17
	408.9	244.1	unique for SDMA	18
OPA-monothioglycerol [a)]	365.0	205.2	unique for SDMA	18
	365.0	244.2	unique for SDMA	18
	365.0	290.1	unique for SDMA	13
	365.0		no unique daughter for ADMA	
OPA-mercaptopropionylglycine [a)]	464.0		no unique daughter ions	
OPA-mercaptopropionylglycine [a)]	420.1		no unique daughter ions	
NDA-mercaptoethanol	369.0		no unique daughter ions	
NDA-monothioglycerol	421.9		no unique daughter ions	
PITC	338.0	46.1	unique for ADMA	47
	338.0	158.1	unique for ADMA	18
	338.0	171.9	unique for SDMA	20
	338.0	175.0	unique for SDMA	38
no derivatisation	203.0	46.3	unique for ADMA	25
	203.0	133.0	unique for SDMA	22
	203.0	172.0	unique for SDMA	48

Table 4.1. Characteristics of selected daughter ions formed by tandem mass spectrometric fragmentation of ADMA and SDMA derivatives. Daughter ions that were produced with an abundancy less than 5% of the most intense fragment are not shown.

[a)] reagents which formed more than one product with ADMA and SDMA.

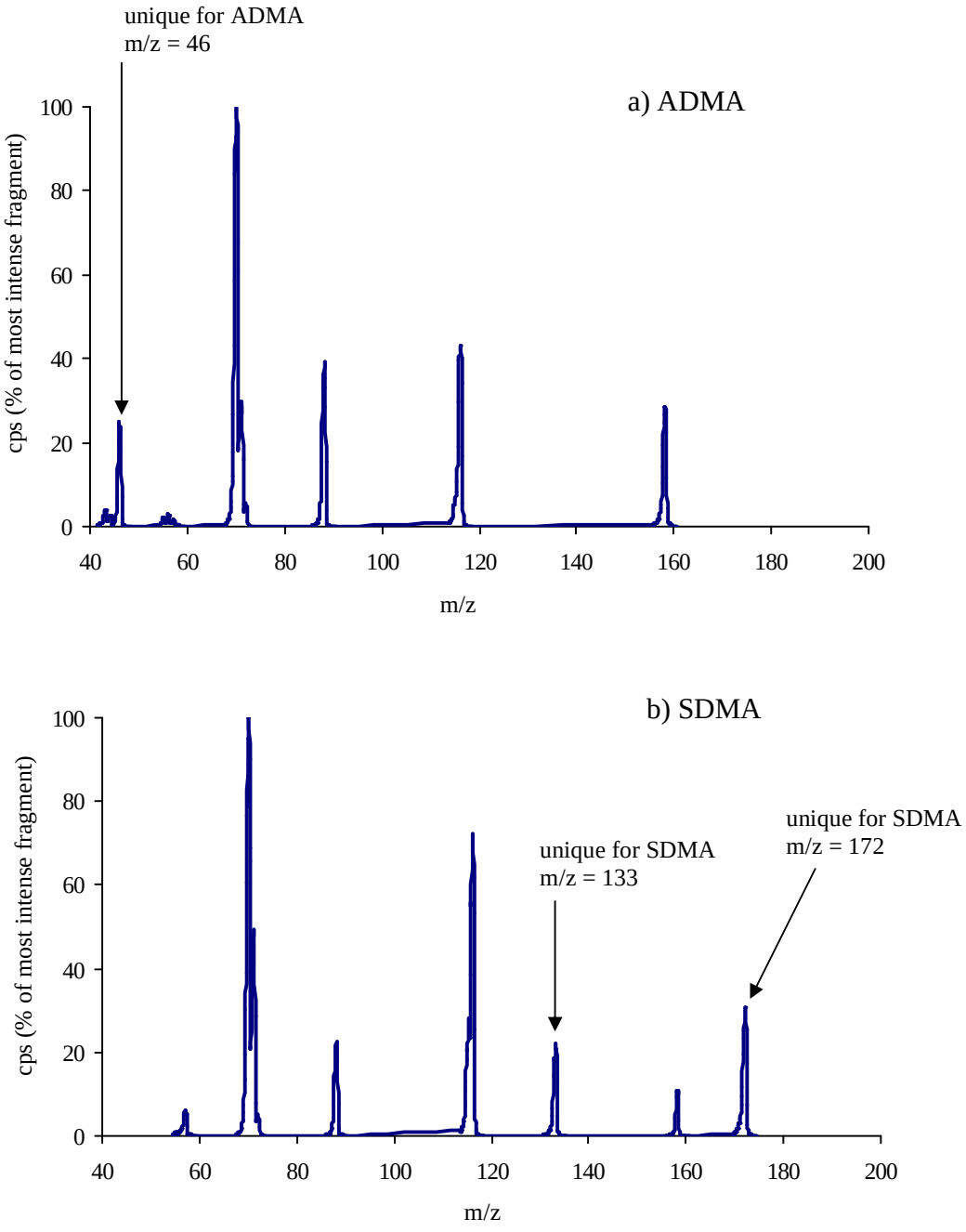


Figure 4.5. The fragmentation patterns of underivatised ADMA and SDMA. a) The transition m/z: 203 → 46 is unique to ADMA. b) The transitions m/z: 203 → 133 and 203 → 172 are unique to SDMA.

4.2. Method Optimisation

4.2.1. Nebuliser Optimisation.

Optimal nebulizer conditions were determined by repeat injection of 10 µl of a 10 µM solution of ADMA and stepwise variation of individual gas flows and temperature. The optimum conditions found were: curtain gas (air) 40 psi, ion source gas 1 (nitrogen) 10 psi, ion source gas 2 (nitrogen) 0 psi, collision gas (nitrogen) 4 psi and temperature 500^{o}C.

4.2.2. Mobile phase optimisation.

Organic solvents such as acetonitrile are often added to mobile phases in order to achieve chromatographic separation of analytes and to improve peak shape, the addition of such solvents can also improve the sensitivity of mass spectrometric methods by enhancing ionisation through increased droplet evaporation in the nebuliser. The effect of mobile phase composition upon ionisation was investigated by using the mass spectrometer HPLC system to mix varying proportions of a) 0.1% formic acid and b) 0.1% formic acid:acetonitrile (50:50) to produce mobile phases with varying concentrations of acetonitrile but constant concentration of formic acid. Repeat 20 µL injections of a standard, consisting of 5 µmol/L of ADMA, SDMA, MMA and arginine, were performed and the concentration of acetonitrile varied in 10% increments from 50% to 0% acetonitrile. Two injections were performed at each acetonitrile concentration before moving on to the next. Finally the sequence of injections was repeated in reverse order of acetonitrile concentration, so as to eliminate possible confounding effects such as a change of detector response with time or a change in the thermal state of the ionisation source. The results of this experiment are shown in figure 4.6. For all four analytes the most intense ionisation was obtained with a mobile phase consisting of 0.1% formic acid, ie with no acetonitrile present. Given the results obtained, concentrations of acetonitrile greater than 50% were not examined and a mobile phase consisting of 0.1% formic acid was chosen for routine use.

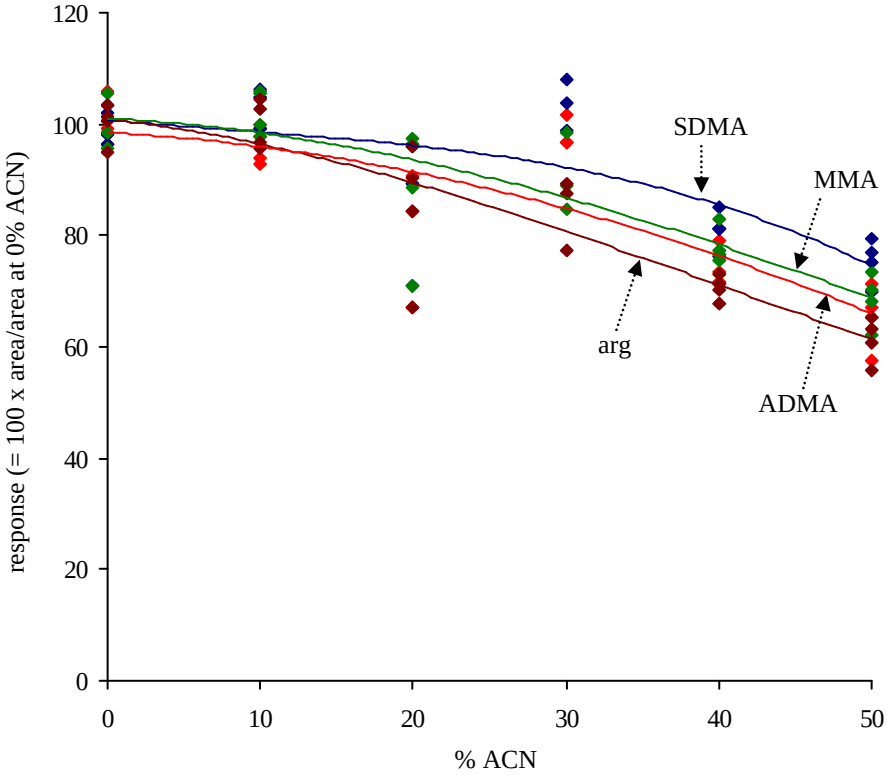


Figure 4.6. The effect of acetonitrile on the ionisation of arginine and methylated arginines. The HPLC system attached to the mass spectrometer was used to mix various proportions of a) 0.1% formic acid and b) acetonitrile/0.1% formic acid. Acetonitrile proportions > 50% were not examined.

4.2.3. Sample preparation

For the analysis of clinical samples tandem mass spectrometry exhibits a very high degree of selectivity in identifying analytes, but specificity cannot be guaranteed due to the possible presence of isobaric interferences. In addition, as discussed previously (see hcy X), the presence of substances other than the analytes of interest in the sample matrix can lead to undesirable signal suppression effects. Interferences and suppression effects may be reduced or eliminated by sample preparation techniques and/or the use of on-line chromatography. Suppression effects may also be reduced by sample dilution. There is a

very large number of possible combinations of sample preparation techniques and chromatographic options which, combined with the selectivity conferred by the mass spectrometer, may produce a working method suitable for the routine analysis of clinical samples. Given these possible combinations, consideration must also be given to the importance of cost, sample preparation time and mass spectrometer analysis time. In the case of methylated arginines sample preparation by simple protein precipitation techniques were not considered an option as experience from developing the method for homocysteine analysis, together with considerations of reported concentrations of plasma ADMA (approximately ten-fold lower than plasma homocysteine) suggested that the resultant sample dilution would not produce a method with a sufficient signal to noise ratio with the equipment available for the accurate and precise determination of plasma methylated arginines. Due to their amphoteric nature amino acids are particularly difficult to extract from aqueous solutions (including plasma and urine) using simple liquid-liquid extraction techniques. Solid phase extraction is a possible alternative and in this case the amphoteric property of amino acids lends itself to solid phase extraction using ion exchange columns. The possibility therefore existed that if solid phase extraction resulted in a sample preparation method with sufficient selectivity then it may be possible to avoid the use of on-line chromatography altogether.

Strong cation SPE columns were obtained from Sigma-Aldrich and included guidelines for their use. These suggested that samples should be diluted in a suitable buffer to ensure that the analytes functional groups are ionised, and that the SPE columns should first be conditioned with either methanol or acetonitrile and then equilibrated with a solution similar to that in which the sample had been diluted. Solvents suitable for washing the columns post sample application included buffers and methanol, and a solution of ammonium hydroxide in methanol was a suitable solvent for the elution of basic compounds from the columns. In following the manufacturer's general guidelines for method development, the extraction procedure detailed in section 2.3. p49, above, was employed.

4.2.4. Possible Interferences and Selectivity of Extraction Procedure.

The following substances were ones which might possibly produce interferences in the method under development: homoarginine, acetyl glutamine, Nα-acetyl lysine and Nε-acetyl lysine (molecular weight 188, isobaric with MMA), tryptophan (molecular weight 202, isobaric with ADMA-d2 and SDMA-d2), and tyrosine (molecular weight 181, isobaric with arginine-d7). To investigate this possibility, 200 μmol/L solutions of each substance (obtained from Sigma-Aldrich) were prepared and analysed, but without the use of solid phase extraction. No interference was observed for tryptophan, homoarginine, acetyl glutamine, Nα-acetyl lysine or Nε-acetyl lysine but tyrosine did produce a fragment, m/z: 182 > 77, isobaric with that used to determine arginine-d_7 (data not shown).

To determine the extent that this might interfere in the quantitation of arginine, tyrosine standards were added to pooled plasma (9 parts plasma to 1 part standard) to increase the endogenous tyrosine concentration by 200, 400, 600, 800 and 1000 μmol/L; this range of tyrosine concentrations greatly exceeds that found in healthy subjects (32 – 88 μmol/L)[200] and pathological conditions excluding tyrosinaemia. These preparations were prepared by solid phase extraction and analysed by mass spectrometry. In addition an aqueous arginine standard, 100 μM, was also spiked with tyrosine and these preparations analysed both including and omitting the solid phase extraction procedure. The results are shown in figure 4.6. If extraction is not used then tyrosine, through isobaric interference, increases the signal produced by arginine-d_7, leading to a decrease in measured arginine concentrations. The solid phase extraction procedure completely removes this interference.

To further investigate the specificity of the extraction step, two standards, (each consisting of a mixture of seven different amino acids), were prepared and analysed separately. Standard A consisted of proline, leucine, lysine, histidine, arginine, tyrosine and monomethylarginine. Standard B consisted of valine, ornithine, methionine, phenylalanine, citrulline, homoarginine and ADMA. These amino acids were chosen as they offered a range of structures and physical characteristics and are commonly found in plasma. The structures and properties of these amino acids are given in table 4.2. The concentration of

each amino acid was 500 µmol/L. Standards were diluted with 0.1 mol/L HCl (1 part standard: 5 parts HCl), and 1 mL of this was applied to the SPE columns, which were conditioned, washed and eluted as described in section 2.3. Each standard was collected upon passing through the SPE column, rather than allowing it to pass to waste, and the ammonia/methanol eluate was collected as normal. The approximate percentage of each amino acid retained by the column, and then recovered from it after washing and eluting are shown in table 4.3.

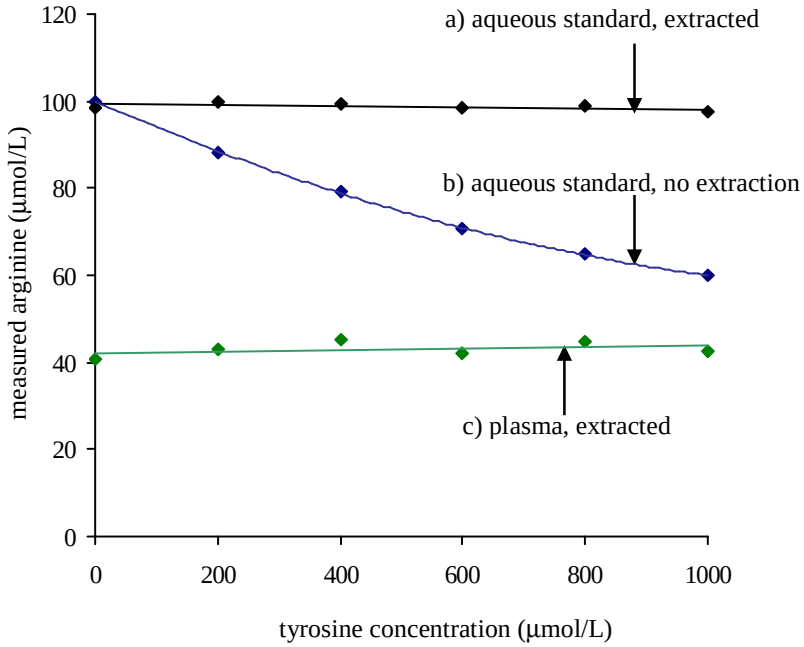


Figure 4.7. The effect of tyrosine on measured arginine, and its elimination by solid phase extraction. A series of aqueous standards were prepared, each containing 100 µmol/L arginine, but increasing concentrations of tyrosine. These were analysed using the methylated arginine method a) as normal, b) omitting the solid phase extraction stage. In addition a plasma sample was spiked with an aqueous tyrosine standard c) and also analysed (with SPE extraction). Tyrosine produces an isobaric interference with the internal standard arginine-d_7, *increasing* its signal in the mass spectrometer and resulting in a *decrease* in measured arginine concentrations. The SPE columns used in this method do not retain tyrosine and eliminate its interference.

Although solid phase extraction with cation exchange columns might be expected to be extremely non-selective, the results of these experiments demonstrate that this is not so and that the converse is in fact true. Of the test substances examined only those that are very basic in nature are completely extracted and eluted from the SPE columns using the protocol described. Amino acids showing some degree of basicity, such as ornithine, lysine and histidine, are partially retained and eluted whereas all other amino acids examined show little extraction by the columns and do not appear in the final eluate.

This experiment was repeated using weak cation exchange columns (also obtained from Sigma). With these columns no extraction of any of the amino acids examined was observed.

4.2.5. Evaluation of Suppression effects.

Experiments to investigate the extent of ion suppression were carried using the solid phase extraction procedure to prepare a) internal standards and b) plasma (without the addition of internal standard). The internal standard extract was reconstituted in carrier solution (0.1% formic acid) and this was then used to reconstitute the plasma extract. The internal standard extract and plasma extract were analysed ten times each and the signals for the internal standards obtained from the two different extracts compared. In the presence of extracted plasma, the signal from each of the internal standards was suppressed by approximately 40%. The degree of suppression was higher than expected, especially given the degree of selectivity conferred by the solid phase extraction step demonstrated above but nevertheless, at this stage, it was considered to be acceptable for further method development.

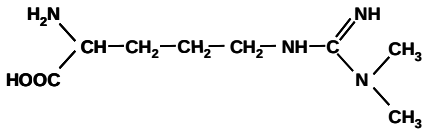
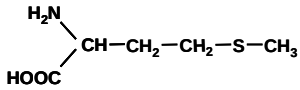
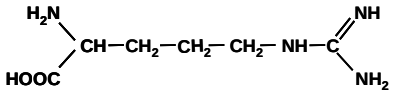
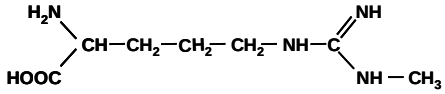
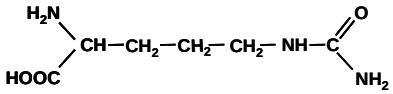
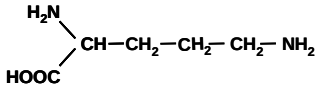
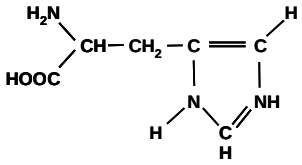
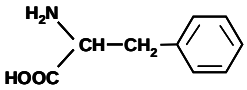
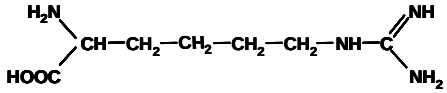
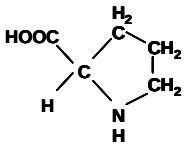
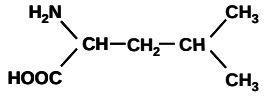
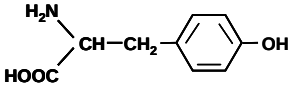
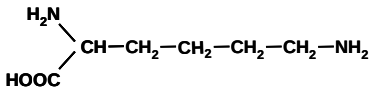
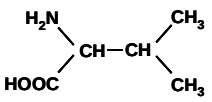
ADMA fw = 202 	methionine fw = 149 pI = 5.7 
arginine fw = 174 pI = 10.8 pK = 12.6 [a)] 	MMA fw = 188 
citrulline fw = 175 	ornithine fw = 132 pK = 10.8 [a)] 
histidine fw = 155 pI = 7.6 pK = 6.0 	phenylalanine fw = 165 pI = 5.9 
homoarginine fw = 188 	proline fw = 115 pI = 6.3 
leucine fw = 131 pI = 6.0 	tyrosine fw = 181 pI = 5.6 pK = 10.1 [a)] 
lysine fw = 146 pI = 9.5 pK = 10.3 [a)] 	valine fw = 117 pI = 6.0 

Table 4.2. The structures and properties of amino acids used to test the retention characteristics of SPE columns[201].
[a)] pK of side chain

	% retained by SPE column	% obtained after washing and eluting column
ADMA	100	100
arginine	75	75
citrulline	20	0
histidine	50	50
homoarginine	100	90
leucine	10	0
lysine	40	30
methionine	60	0
MMA	90	90
ornithine	60	50
phenylalanine	30	0
proline	5	0
tyrosine	10	0
valine	20	0

Table 4.3. The retention and elution behaviour of Supelclean LC-SCX strong cation exchange columns with selected amino acids.

4.2.6. Linearity

To assess linearity five aqueous standards were prepared (ADMA, SDMA: 5, 10, 15, 20 and 25 μmol/L, methylarginine: 10, 20, 30, 40 and 50 μmol/L and arginine: 50, 100, 150, 200, and 250 μmol/L). Each standard was analysed in triplicate in random order. Inspection of the standard curves obtained showed linearity for the range of standards (figure 4.7). The regression data for the standard curves is given in table 4.3.

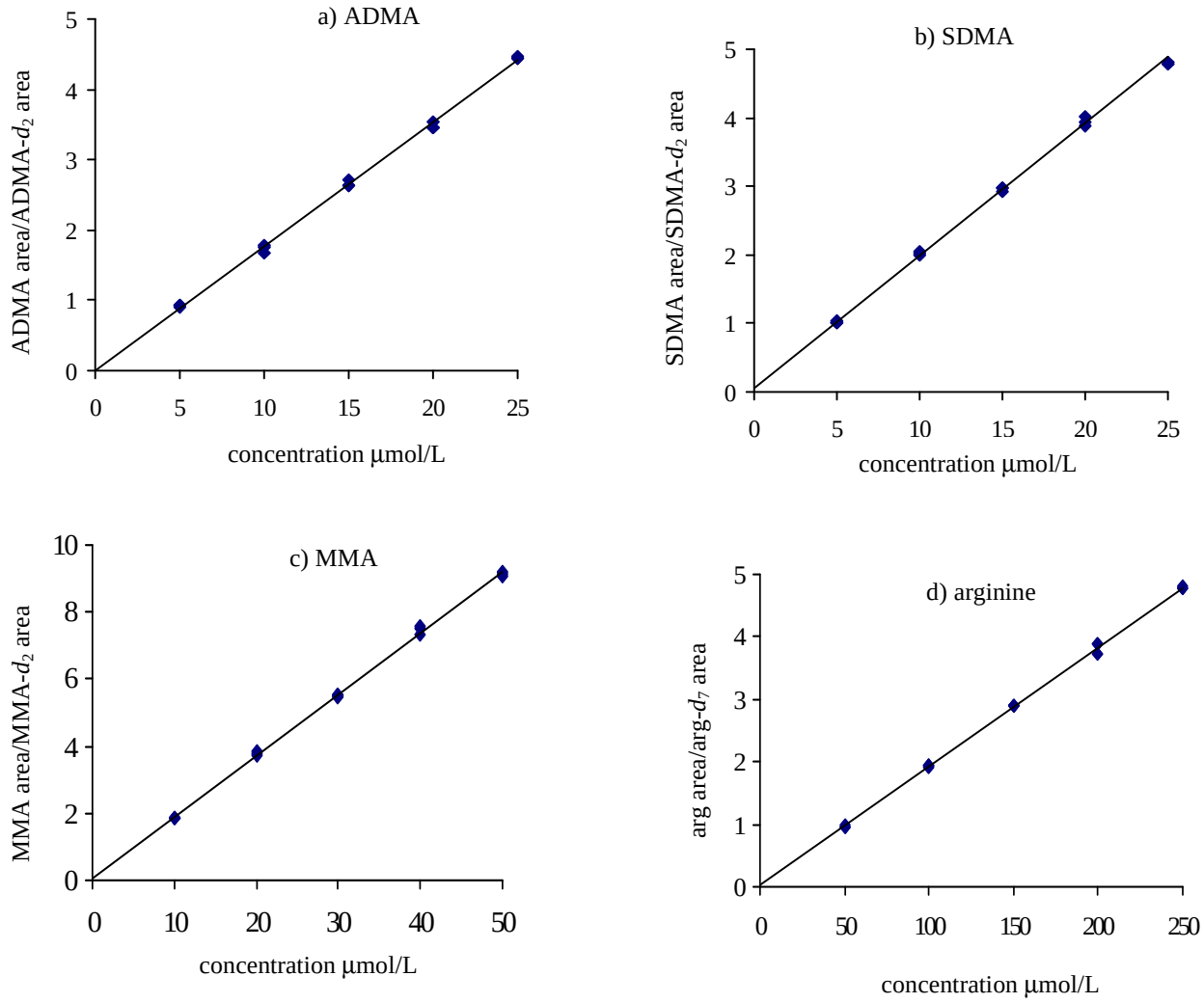


Figure 4.8. Standard curves for ADMA, SDMA, MMA and arginine. The results for each standard are plotted individually, but many points are coincidental.

	standards (n)	slope	sd slope	intercept	sd intercept	standard error
ADMA	15	0.1722	0.0019	0.0406	0.0312	0.0516
SDMA	15	0.1955	0.0014	0.0670	0.0237	0.0391
MMA	15	0.1823	0.0017	0.0639	0.0568	0.0938
arg	15	0.0183	0.0001	0.0235	0.0230	0.0379

Table 4.4. Regression data for ADMA, SDMA, MMA and arginine standard curves.

4.2.7. Precision

Within and between batch precision was assessed by analysing plasma and urine samples, at three different concentrations, ten times each. For low concentrations pooled plasma or pooled urine was used. For intermediate and high concentrations the low concentration pools were spiked with aqueous standard (pool:standard::19:1).

Results are shown in table 4.4 In addition the results obtained for a normal plasma sample are shown in figure 4.8.

Sample type	analyte	Within-batch		Between-batch	
		Mean (μmol/L)	%CV	Mean (μmol/L)	%CV
Plasma	ADMA	0.497	1.7	0.499	4.1
		1.411	2.1	1.486	2.6
		5.637	0.9	5.693	1.8
	SDMA	0.547	2.2	0.515	7.6
		1.338	2.4	1.335	3.0
		4.931	1.7	4.825	1.8
	MMA	0.158	3.0	0.153	13.5
		2.280	0.9	2.403	2.5
		11.560	0.6	11.950	3.0
	arginine	109	1.1	114	1.4
		125	1.1	133	2.2
		237	1.2	239	1.9
Urine	ADMA	86.2	2.0	85.6	2.1
		102.4	2.0	102.2	2.6
		185.7	2.0	182.9	2.2
	SDMA	68.8	2.7	69.8	3.2
		84.9	2.0	87.0	3.0
		163.7	2.2	166.9	3.3
	MMA	2.1	2.9	1.7	10.8
		22.5	1.9	22.9	2.0
		104.8	2.0	106.5	2.4
	arginine	48.2	5.6	39.0	12.7
		72.5	2.5	62.7	6.0
		169.0	3.1	165.5	7.1

Table 4.5. Within-batch and between-batch precision for plasma and urine ADMA, SDMA, MMA and arginine. Ten replicates were analysed at each concentration.

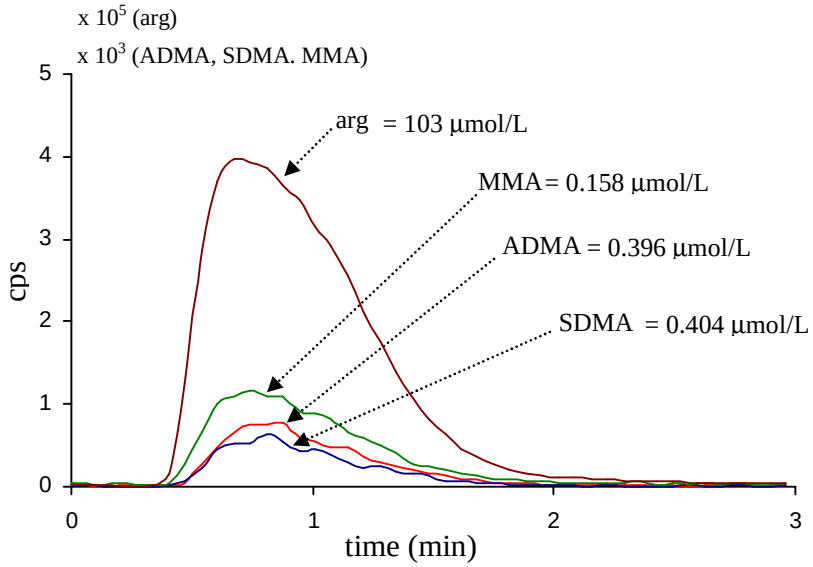


Figure 4.9. The mass-spectrogram of a typical plasma sample. For purposes of clarity the internal standards are not shown.

4.2.8. Recovery

Recovery experiments were performed by spiking ten individual plasma and ten individual urine samples with either 0.1 mol/L HCl or aqueous standard at two different concentrations (sample: standard::19:1). The results of this experiment are given in table 4.5.

4.2.9. Limits of Detection and Quantitation

The limit of detection and limit of quantitation were determined by analysing ten sample blanks (water in place of sample). The signal (baseline) obtained from each analysis was integrated using Analyst-NT software to obtain a measure of noise. The limit of detection was then calculated as 3 x blank sd and the limit of quantitation as 10 x blank sd.

The limits of detection for ADMA, SDMA, MMA and arginine were 0.023 µmol/L, 0.011 µmol/L, 0.009 µmol/L, and 0.258 µmol/L respectively, while the limits of quantitation were 0.077 µmol/L, 0.037 µmol/L, 0.030 µmol/L and 0.860 µmol/L respectively.

Sample type	Analyte	Added (μmol/L)	Mean recovery (%)	%CV
Plasma	ADMA	1	91.7	10.3
		5	91.4	2.2
	SDMA	1	91.7	11.8
		5	91.4	4.0
	MMA	2	90.3	5.8
		10	91.6	1.5
	arginine	20	98.2	23.2
		100	90.5	4.3
Urine	ADMA	20	91.7	19.5
		100	92.0	5.6
	SDMA	20	98.0	23.2
		100	93.5	5.9
	MMA	20	98.3	6.1
		100	98.3	4.6
	arginine	20	89.4	14.3
		100	92.6	4.3

Table 4.6. Recovery of ADMA, SDMA, MMA and arginine from plasma and urine. Baseline samples were prepared by the addition of 0.1 mol/L HCl, spiked samples by the addition of standard in 0.1 mol/L HCl (1 part HCl or standard to 19 parts sample), Ten samples were prepared at each concentration.

4.2.10. Plasma and urine results.

To verify that the method would produce results similar to those described in the literature, lithium heparin plasma from nine healthy volunteers was analysed (table 4.6) and the results obtained were comparable to values reported in other studies (table 4.7). Five twenty-four hour urine collections, surplus samples from patients undergoing routine investigations, but with no indications of vascular disease, were also analysed (table 4.8). There is little data available for urine concentrations and outputs of arginine and methylated arginines, but in a study by Huang et al similar values were reported for ADMA and SDMA, however in that publication urinary arginine outputs were considerably higher than those found here (mean urinary arginine 114 ± 30 vs 12.7 ± 8.5 μmol/24hr).

	Age (years)	ADMA (μmol/L)	SDMA (μmol/L)	MMA (μmol/L)	arg (μmol/L)
	24	0.512	0.472	0.182	132
	18	0.177	0.448	0.201	249
	24	0.265	0.504	0.157	167
	20	0.290	0.414	0.332	316
	24	0.387	0.371	0.158	125
	20	0.484	0.523	0.167	125
	18	0.443	0.547	0.195	150
	19	0.627	0.456	0.190	133
	21	0.372	0.499	0.176	64
mean ± 1sd	20.9 ± 2.5	0.395 ± 0.139	0.470 ± 0.055	0.195 ± 0.054	162 ± 76

Table 4.7. Concentrations of arginine and methylated arginines in plasma of nine healthy male volunteers.

Analyte	Range of mean concentrations reported (μmol/L)	Median of mean concentrations reported (μmol/L)
ADMA	0.30 - 2.60	1.04
SDMA	0.34 - 3.0	0.73
MMA	0.100 - 0.177	0.104
arginine	50 - 236	110

Table 4.8. Concentrations of plasma ADMA, SDMA, MMA and arginine reported in published studies. Adapted from Zhang et al[150]

Age (years)	Urine volume (ml)	ADMA (μmol//24hr)	SDMA (μmol/24hr)	MMA (μmol/24hr)	arg (μmol/24hr)
59	1233	48.8	42.5	1.55	20.8
25	2594	57.6	62.3	1.85	6.0
41	2466	52.3	59.7	1.18	22.9
52	2714	34.2	58.1	1.35	5.6
35	2150	46.2	40.0	1.34	7.9
mean ± 1sd		47.8 ± 8.7	52.5 ± 10.4	1.5 ± 0.3	12.7 ± 8.5

Table 4.9. Concentrations of arginine and methylated arginines in five 24 hour urine collections.

4.3. Investigation of sample stability

To investigate the effects of delayed separation of plasma from red-cells upon homocysteine, cysteine, methionine, arginine and methylated arginine concentrations 7 mL of lithium heparin anticoagulated blood samples were collected from each of five volunteers, on five separate occasions. Approximately 1 mL of whole blood was removed from each tube immediately upon blood collection, centrifuged at 1000g for five minutes, the plasma was then removed from the red-cells and frozen at -20^{O}C. The tubes were left to stand at room temperature (21^{O}C), and at timed intervals (1, 2, 4, 8, and 24 hours) the tubes were mixed by inversion, a further 1 ml of blood removed, centrifuged and the plasma again removed and stored at -20^{O}C until analysis. Samples were analysed for total homocysteine, total cysteine and methionine within one batch and also for arginine and the methylated arginines within one batch. All analyses were performed on the same day to avoid repeat freeze thawing of samples. For total cysteine and methionine no statistically significant difference was detected for samples separated 24 hours post blood collection as compared with immediate separation (Student's paired t-test: $p = 0.250$, and 0.157 respectively). For total homocysteine plasma concentrations increased gradually over the 24 hour period, consistent with previous observations that red-cells continue to produce homocysteine post blood collection[53], however no statistically significant difference was detected for samples separated 2 hours post blood collection as compared with immediate separation (Student's paired t-test: $p = 0.08$). For ADMA, SDMA and MMA no statistically significant difference was detected for samples separated 8 hours post blood collection as compared with immediate separation (Student's paired t-test: $p = 0.277$, 0.667 and 0.096 respectively) however significant differences were seen for samples separated 24 hours post collection with ADMA being on average 28% higher than samples separated immediately, SDMA 10% higher and MMA 47% higher. The concentration of arginine was significantly lower at each time interval compared to samples separated immediately (figure 4.9), with concentrations at 1 hour post separation being on average 17% lower than samples separated immediately and this is most probably due to transport of arginine into red-cells with subsequent metabolism to ornithine by red-cell arginase[202].

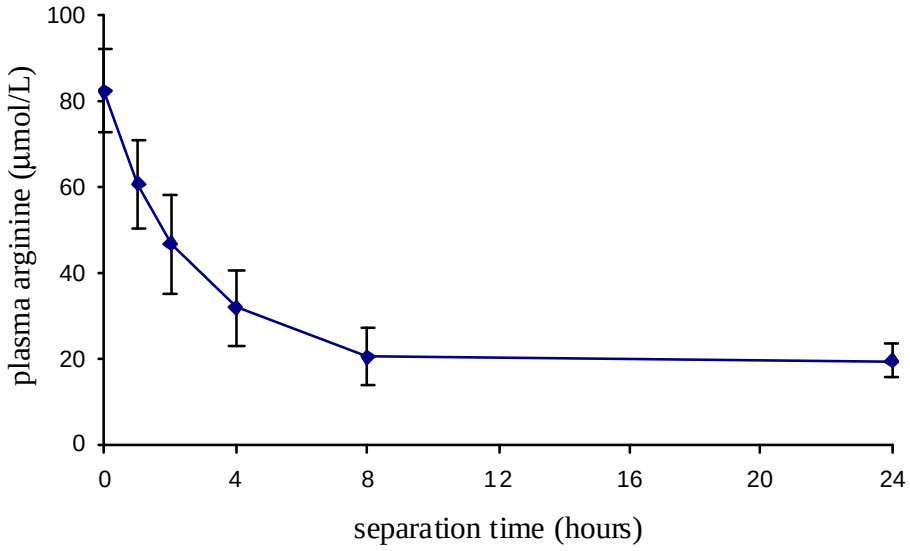


Figure 4.10. The effect of delayed separation on plasma arginine. Five lithium heparin anti-coagulated blood samples were stored at room temperature, and at timed intervals 1 mL of blood was removed, the plasma separated, and stored at -20^{O}C until analysis. The results shown are mean ± 1 sd.

In conclusion for arginine analysis plasma must be removed from red-cells immediately upon blood collection, for homocysteine analysis separation should take place within two hours whereas for ADMA, SDMA, MMA, cysteine and methionine lithium heparin anticoagulated whole blood samples are stable for at least 8 hours.

4.4. Conclusion

A method for the simultaneous analysis of asymmetric dimethylarginine, symmetric dimethylarginine, monomethylarginine and arginine in human plasma and urine, with short analysis time and isotopic internal standardisation for each analyte has been described. The method requires neither sample derivatisation nor the need for chromatographic separation of analytes. The method described shows good precision and accuracy and is suited for both research purposes and implementation in the busy, routine clinical laboratory. In addition the synthesis and utilisation of isotopically labelled symmetric dimethylarginine and monomethylarginine is described for the first time, avoiding the use of surrogates such as homoarginine for internal standardisation.

5. Homocysteine and Methylated Arginines in Clinical Samples

5.1. Investigations With Routine Clinical Samples

5.1.1. Sample Selection

The methods for homocysteine and methylated arginines developed in Chapters 3 & 4 were applied to the analysis of routine plasma samples to determine whether plasma total homocysteine concentration is a determinant of plasma ADMA concentration, and in addition to explore what relationships might exist between total homocysteine, methylated arginines, patient demographics such as age and gender, and other routinely assessed biochemical parameters. Plasma arginine was not included part of these studies, as it could not be guaranteed that plasma had been separated from cells sufficiently promptly to allow for its reliable measurement. The samples analysed consisted of a random and essentially heterogeneous selection of 111 specimens that had been received by the laboratory for routine homocysteine analysis. The clinical details that had been given with these requests included cardiovascular disease, raised lipids, pulmonary embolism, secondary prevention, various miscellaneous comments unrelated to any form of vascular disease and were often missing. In addition 9 samples from patients with hypothyroidism (TSH > 5 mu/L) were included as well as 14 patients with a hyperthyroid pattern of results (TSH < 0.3 mu/L). All work was performed in accordance with local ethical guidelines.

Patient samples had been collected by venepuncture into Greiner Vacuette tubes containing lithium heparin as anticoagulant. After centrifugation at 1000 g for 10 minutes plasma was removed from the samples and stored at -20 ^{o}C until being prepared for homocysteine analysis at which point they were refrozen, and again stored at -20 ^{o}C until being tested for methylated arginines and the remaining biochemical parameters:- sodium, potassium, creatinine, urea, total protein, albumin, cholesterol, uric acid, TSH and fT4. Serum B12 and folate were recorded where this had formed part of the original sample request but was not tested for otherwise, as serum samples, paired with the plasma samples, were generally unavailable and plasma samples were not considered suitable for folate measurements by

the manufacturer of the folate kit. All analytes other than homocysteine and methylated arginines were measured on a Roche Modular system, the methods used and associated reference ranges are given in table 5.1. Samples were anonymised other than the recording of gender, age and clinical information.

Analyte	Method	Reference range (at Royal Sussex County Hospital, Brighton)
Na	indirect ion-selective electrode	135 - 146 mmol/L
K	indirect ion-selective electrode	3.2 - 5.1 mmol/L
creatinine	colorimetric using alkaline picric acid (Jaffe reaction)	Females: 44 - 80 μmol/L Males: 62 - 106 μmol/L
urea	enzymatic colorimetric	1.7 - 8.3 mmol/L
total protein	colorimetric using biuret	66 - 87 g/L
albumin	colorimetric using bromocresol green	34 - 48 g/L
uric acid	enzymatic colorimetric using uricase	Females: 0.14 - 0.34 mmol/L Males: 0.20 - 0.42 mmol/L
B12	immunoassay with electrochemiluminescent detection	197 - 866 pg/mL
folate	competitive binding assay with electrochemiluminescent detection	4.6 - 18.7 ng/mL
cholesterol	enzymatic colorimetric using cholesterol oxidase	< 5.0 mmol/l
TSH	immunoassay with electrochemiluminescent detection	0.3 - 4.2 mU/L
fT4	immunoassay with electrochemiluminescent detection	12 - 22 pmol/L

Table 5.1. Methods used, and reference ranges for, biochemical parameters measured in clinical studies.

5.1.2. Preliminary Investigations, Calculations and Data Transformations

5.1.2.1. Gender

The combined data from groups all three groups was initially investigated to see if any variables showed differences between the genders. No assumptions were made about the underlying data structure and the non-parametric Mann-Whitney U test was used for the

analysis. The data set was well balanced in the proportions of males and females (males 47.8%). All data analyses were performed using SPSS 15.0.

Significant gender differences were found for age ($p = 0.007$), creatinine ($p < 0.001$), uric acid ($p < 0.001$) and urea ($p < 0.001$). Significant differences ($p <= 0.05$) were not found for any other variable.

5.1.2.2. Age

The median age for females in the combined group was 48.5 years while for males it was 58.0 years. Inspection of the subgroups showed that this difference in age was most likely to be due to a selection bias (figure 5.1). Although the samples in the homocysteine group had mostly be selected at random, only a few samples had been available with hyperthyroid and hypothyroid results and these had been dominated by samples from comparatively young females (hyperthroid: median age = 45 yr, n = 11, hypothyroid: median age = 47 yr, n = 6) with only a few samples from comparatively older males (hyperthroid: median age 64 yr, n = 3, hypothyroid: median age = 79 yr, n = 3). There was no statistically significant difference between genders for age when considering only the homocysteine sample group ($p = 0.094$).

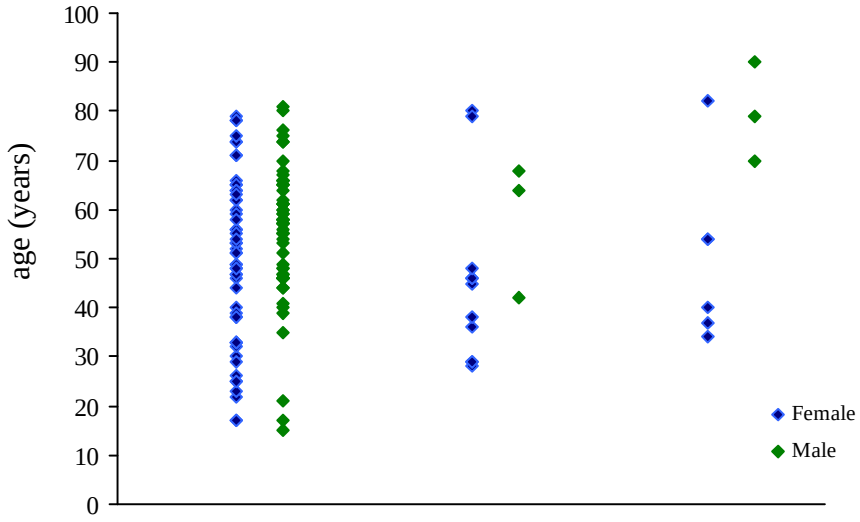


Figure 5.1. The age distribution of patients by subgroup.

5.1.2.3. Plasma creatinine and uric acid.

Both plasma creatinine and uric acid show a strong association with gender and most laboratories quote a gender related reference range for each of these analytes. In order to remove the gender association and simplify data analysis a transform was applied to both plasma creatinine and uric acid results to express them in terms of their difference from the mid-point of the reference range, in terms of standard deviations (figures 5.2. and 5.3).

$$\text{transformed result} = \frac{\text{plasma result - mid-point reference range}}{\text{sd}}$$

$$\text{where sd} = \frac{\text{upper reference range limit - lower reference range limit}}{4}$$

For plasma creatinine (μmol/L):

Females:	reference range	=	44 - 180,	mid-point = 62,	sd = 9
Males:	reference range	=	62 - 106,	mid-point = 84,	sd = 11

For plasma uric acid (mmol/L):

Females:	reference range	=	0.14 - 0.34,	mid-point = 0.24,	sd = 0.050
Males:	reference range	=	0.20 - 0.42,	mid-point = 0.31,	sd = 0.055

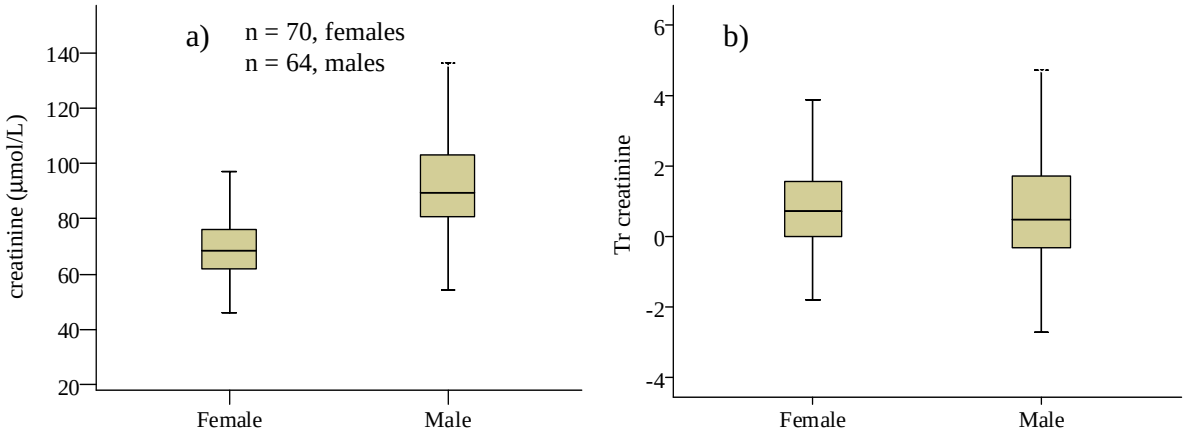


Figure 5.2. The elimination of gender associated differences in plasma creatinine by data transform. a) Plasma creatinine shows a significant difference between genders (Mann-Whitney U, p <0.001) b) there is no significant difference between genders after applying the data transform described in section 5.1.2.3 (Mann-Whitney U, p = 0.596).)

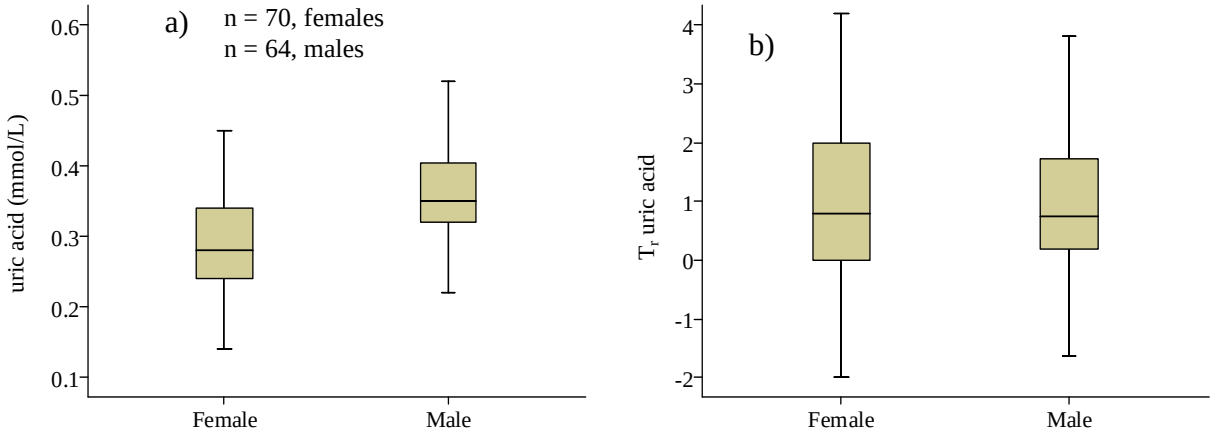


Figure 5.3. The elimination of gender associated differences in plasma uric acid by data transform. a) Plasma uric acid shows a significant difference between genders (Mann-Whitney U, p <0.001) b) there is no significant difference between genders after applying the data transform described in section 5.1.2.3 (Mann-Whitney U, p = 0.897).)

5.1.2.4. Urea

The reference range for plasma urea (1.7 - 8.3 mmol/l) in use at RSCH is not stratified according to either age or gender however, examination of the data showed a significant correlation between urea and age (Spearman's rho <0.001) and linear regression analysis produced the following equations:

females, urea = 3.47 + 0.029 age
males, urea = 4.27 + 0.032 age

The rate of increase of urea with age was approximately the same for both females and males at approximately 0.03 mmol/L/yr (figure 5.4).

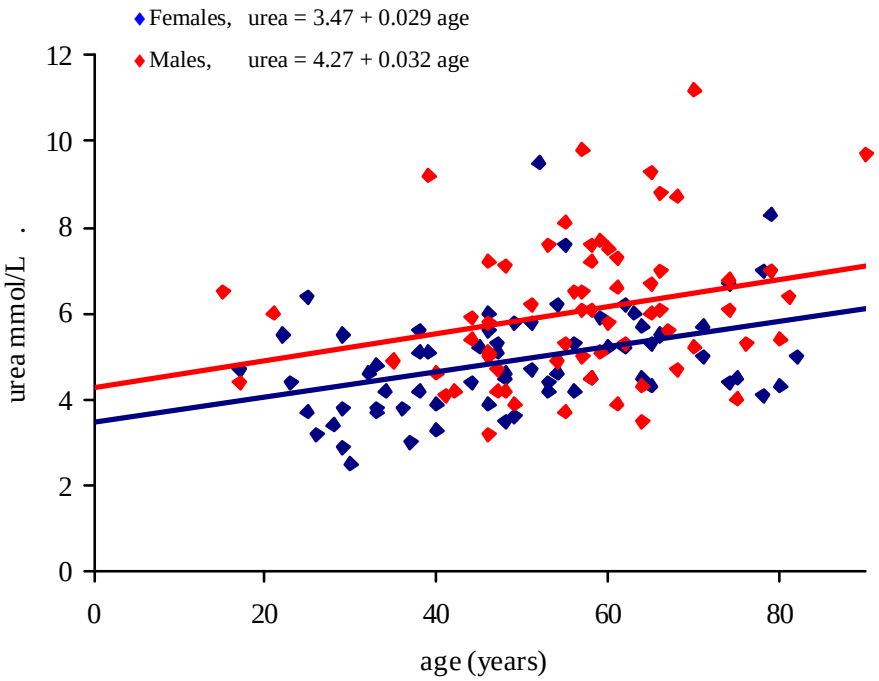


Figure 5.4. Change of plasma urea with age. Urea was significantly associated with age for both females (Pearson correlation: r = 0.407, p < 0.001) and males (Pearson correlation: r = 0.265, p = 0.035) For both males and females plasma urea changes by approximately 0.03 mmol/L/year.

To test whether the difference in plasma urea between the sexes that had been identified in the data was accounted for by the change of urea with age, in conjunction with the age selection bias noted above, an 'age adjusted urea' was calculated according to the following formula:

$$\text{age adjusted urea} = \text{measured urea} - 0.03\ \text{age}.$$

and this new variable, grouped by gender, was then subjected to the Mann-Whitney U test. A statistically significant difference between the sexes was detected for age adjusted urea and it was concluded that further analysis involving plasma urea may require separate analysis by gender.

5.1.2.5. Tests for Normality

All variables (except gender) were examined for normality using the Kolmogorov-Smirnov test and all passed this test with the exception of total homocysteine ($p < 0.001$), SDMA ($p = 0.007$), albumin ($p < 0.001$) and TSH ($p < 0.001$). A logarithmic transform was found to normalise both total homocysteine ($p = 0.196$) and also SDMA ($p = 0.174$) (figures 5.5. and 5.6). Albumin could not be normalised using simple transforms (logarithmic, power) and so relationships between albumin and other variables were examined nonparametrically. The lack of normality for TSH was attributed to the way in which samples had been selected, as TSH was found to conform to a normal distribution when the three subgroups were tested individually.

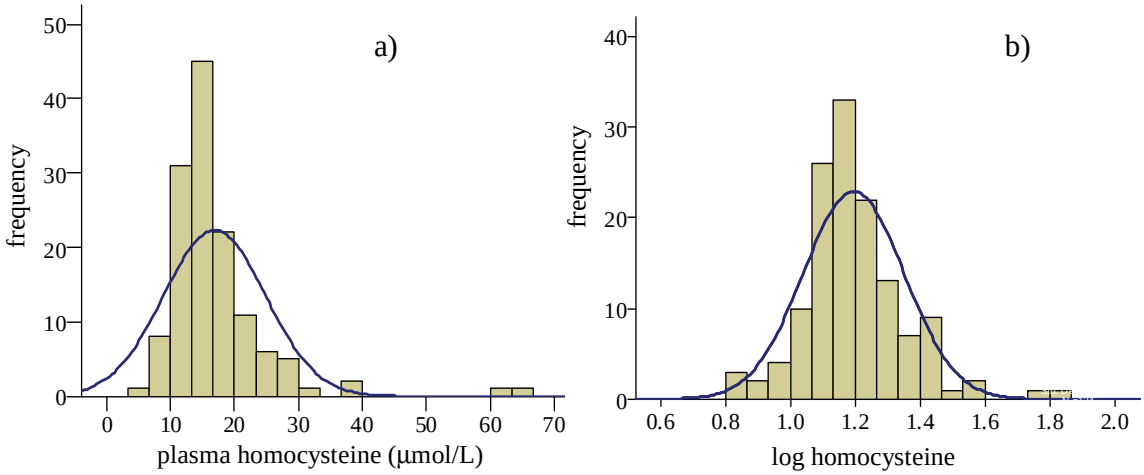


Figure 5.5. The effect of log transform on plasma total homocysteine results. a) Plasma homocysteine did not pass the Kolmogorov-Smirnov test for normality ($p < 0.001$) but following a log transformation b) the results were effectively normalised ($p = 0.196$)

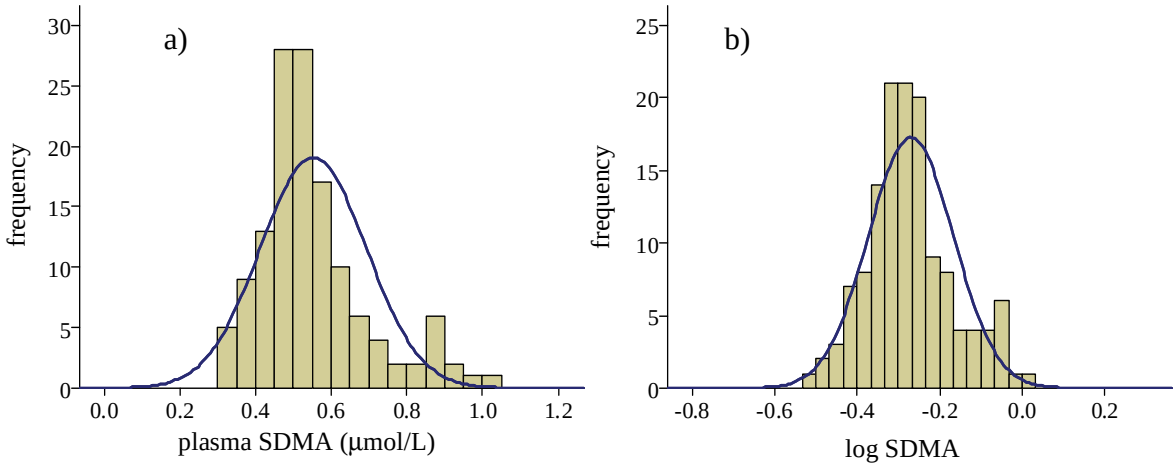


Figure 5.6. The effect of log transform on plasma SDMA results. a) Plasma SDMA did not pass the Kolmogorov-Smirnov test for normality ($p < 0.001$) but following a log transformation b) the results were effectively normalised ($p = 0.174$)

5.1.2.6. Biochemical results for sample groups and associations between variables

The results of biochemical analyses for individual subgroups, and all groups combined, are shown in table 5.1, and the correlations between variables are shown in table 5.2.

		Routine homocysteine group	Hypothyroid group	Hyperthyroid group	Combined group
n		111	14	9	134
% male		52.3	21.4	33.3	47.8
Age (years)	mean	52.8	48.4	60.0	52.8
	sd	15.55	17.67	20.98	16.20
Na (mmol/L)	mean	143.0	141.9	140.3	142.7
	sd	2.79	3.51	4.95	3.10
K (mmol/L)	mean	4.27	4.34	4.22	4.28
	sd	0.408	0.348	0.511	0.408
creatinine (μmol/L)	mean	83.24	66.29	78.33	81.14
	sd	19.54	16.264	25.426	20.195
T_r creatinine [a)]	mean	0.974	-0.11	1.22	0.866
	sd	1.5044	-1.17 - 1.36	-1.12 - 2.78	1.5845
urea (female) (mmol/L)	mean	4.99	4.90	4.38	4.92
	sd	1.221	1.385	1.170	1.237
urea (male) (mmol/L)	mean	6.09	4.13	7.30	6.06
	sd	1.660	0.603	2.265	1.708
total protein (g/L)	mean	76.5	76.2	72.4	76.2
	sd	4.65	5.48	7.38	5.02
albumin (g/L)	median	45.0	44.0	40.0	45.0
	IQR	43.0 - 46.0	42.5 - 46.0	34.0 - 44.0	43.0 - 46.0
uric acid (mmol/L)	mean	0.330	0.303	0.268	0.323
	sd	0.0734	0.0911	0.0940	0.0780
T_r uric acid [b)]	mean	1.025	0.914	0.054	0.948
	sd	1.2775	1.3667	1.3558	1.3046

Table 5.2. The results of biochemical analyses for clinical samples.
Continued on next page.

[a)] T_r creatinine = transformed creatinine
[b)] T_r uric acid = transformed uric acid

		Routine homocysteine group	Hypothyroid group	Hyperthyroid group	Combined group
n		111	14	9	134
cholesterol (mmol/L)	mean sd	4.83 1.224	4.75 1.015	4.91 1.484	4.83 1.214
TSH (mU/L)	median IQR	1.81 1.17 - 2.29	0.01 0.01 - 0.04	12.60 8.39 - 39.21	1.76 1.02 - 2.50
log TSH	median IQR	0.258 0.068 - 0.360	-2.000 -2.000 - -1.398	1.100 0.914 - 1.590	0.244 0.008 - 0.398
fT4 (pmol/L)	mean sd	15.88 2.318	23.36 5.053	10.44 3.318	16.29 3.9129
total homocysteine (µmol/L)	median IQR	14.8 12.9 - 18.5	13.9 11.9 - 16.0	19.0 13.9 - 23.5	14.9 12.9 - 18.5
log total homocysteine	mean sd	1.197 0.1626	1.153 0.1010	1.251 0.1281	1.196 0.1557
ADMA (µmol/L)	mean sd	0.4987 0.08278	0.5923 0.12119	0.4707 0.07529	0.5066 0.09141
SDMA (µmol/L)	median IQR	0.526 0.470 - 0.608	0.528 0.403 - 0.570	0.501 0.460 - 0.777	0.527 0.459 - 0.607
log SDMA	mean sd	-0.2686 0.10167	-0.297 0.1032	-0.243 0.1252	-0.2699 0.10324
MMA (µmol/L)	mean sd	0.1252 0.03498	0.1691 0.03856	0.1134 0.03010	0.1290 0.03755

Table 5.2. The results of biochemical analyses for clinical samples.
Continued from previous page.

		log thcy	ADMA	log SDMA	MMA
log thcy	Correlation Coefficient		0.197	0.252	0.069
	Significance		0.023 *	0.003 **	0.430
ADMA	Correlation Coefficient	0.197		0.315	0.518
	Significance	0.023 *		<0.001 **	<0.001 **
log SDMA	Correlation Coefficient	0.252	0.315		0.225
	Significance	0.003 **	<0.001 **		0.009 **
MMA	Correlation Coefficient	0.069	0.518	0.225	
	Significance	0.430	<0.001 **	0.009 **	
age	Correlation Coefficient	0.206	0.190	0.494	0.112
	Significance	0.017 *	0.028 *	<0.001 **	0.199
Na	Correlation Coefficient	0.151	0.277	0.065	0.022
	Significance	0.082	0.001 **	0.453	0.801
K	Correlation Coefficient	-0.074	-0.089	0.042	0.045
	Significance	0.393	0.306	0.630	0.604
T_r creat	Correlation Coefficient	0.144	-0.010	0.559	0.043
	Significance	0.096	0.906	<0.001 **	0.624
urea female	Correlation Coefficient	0.276	0.205	0.550	0.199
	Significance	0.021 *	0.089	<0.001 **	0.098
urea male	Correlation Coefficient	0.017	0.050	0.369	0.015
	Significance	0.895	0.695	0.003 **	0.904
total protein	Correlation Coefficient	-0.027	-0.186	-0.153	-0.151
	Significance	0.757	0.031 *	0.078	0.081
albumin	Correlation Coefficient [a]	-0.113	-0.164	-0.046	-0.195
	Significance	0.195	0.059	0.601	0.024 *
T_r uric acid	Correlation Coefficient	0.178	0.278	0.360	0.111
	Significance	0.040 *	0.001 **	<0.001 **	0.200
cholesterol	Correlation Coefficient	0.040	-0.131	0.051	-0.054
	Significance	0.650	0.132	0.556	0.538
fT4	Correlation Coefficient	-0.047	0.247	-0.247	0.195
	Significance	0.590	0.004 **	0.004 **	0.024 *
log TSH	Correlation Coefficient [a]	0.157	-0.180	0.248	-0.055
	Significance	0.070	0.037 *	0.004 **	0.529
B12	Correlation Coefficient	-0.181	-0.150	-0.114	-0.284
	Significance	0.472	0.553	0.654	0.253
folate	Correlation Coefficient	-0.290	0.002	0.084	0.378
	Significance	0.244	0.993	0.741	0.122

Table 5.3. Correlations between the results obtained for clinical samples.

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
[a] Spearman correlation coefficient

5.1.3. ADMA and Homocysteine

A plot of ADMA versus log total homocysteine is shown in figure 5.6. The data is highly scattered and the correlation coefficient small (Pearson = 0.197) but statistically significant (p = 0.023). The association between the two could be (i) a random statistical finding, (ii) might be due to a common association with another variable or (iii) might be real and possibly diminished by the effect of other variables.

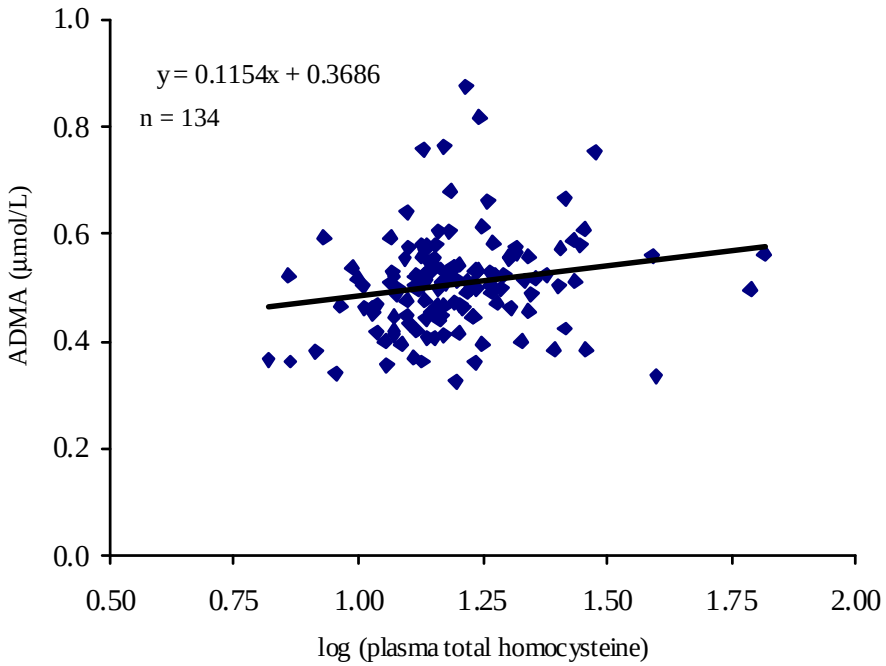


Figure 5.7. The association between plasma ADMA and log (plasma total homocysteine). ADMA and log total homocysteine showed a statistically significant association (Pearson correlation: r = 0.197, p =0.023).

Calculating partial correlation coefficients, allowing for control by other variables, revealed that the association between ADMA and total homocysteine was likely to be due, at least in part, to common associations with age and with T_r uric acid. When age and T_r uric acid were used together as control variables no statistically significant association was detected

between ADMA and log total homocysteine table 5.3. It should be noted that age and T_r uric acid were not correlated (Pearson: p = 0.228).

Control variable(s)	partial correlation coefficient	significance (p)
none	0.197	0.023
age	0.164	0.059
T_r uric acid	0.156	0.073
age and T_r uric acid	0.128	0.145

Table 5.4. Correlation between ADMA and log total homocysteine controlling for other variables. Although a statistically significant association existed between ADMA and log homocysteine ($p <= 0.05$), this no longer remained significant when other variables were taken into account ($p > 0.05$)

5.1.4. Investigation of Data Using Linear Modelling

Linear modelling techniques were used to further investigate the extent to which variables were predictors of plasma ADMA, SDMA and MMA concentrations. The variables included in the models were those which had shown a significant correlation with ADMA; creatinine was also included, although having shown no association with ADMA so far, as a measure of renal function. Two models were used, a standard multivariate least-squares model (in which all variables are included) and a step-wise least squares model (in which variables are entered one at a time, in order of strength of association, until either all variables are entered or there is no association with the remaining variables). The differences between the two techniques were minor and made no difference to the interpretation of the findings, only the results of the step-wise model will therefore be considered. The models were applied to the combined set of results and also to sub-groups by gender and/or thyroid status. Whilst analysing male and female sub-groups urea was included as a variable; no association was seen with ADMA. An association between urea and log SDMA was observed but as this was duplicated by a similar association between creatinine and log SDMA, the inclusion of urea was thought to be redundant and it was

therefore not considered further. The effects of the transforms on creatinine and uric acid were explored by analysing the results for males and females separately, first using the original variables and then using the corresponding transformed variables. Virtually identical results, for all variables in the model, were obtained. The largest difference observed was for the association between ADMA and creatinine for the male subgroup: the significance of the association was 0.425 when using creatinine, and 0.423 when using transformed creatinine and these findings validated the use of the transforms as a means of amalgamating the results for males and females into one group. The results of the regression modelling for all results combined, and selected sub-groups, are shown in table 5.4. With the exception of fT4 the results are generally consistent regardless of the sub-group; some (small) differences are seen but this is not surprising when performing multiple statistical tests (type I and type II statistical errors). fT4 shows a significant association with ADMA, and with MMA, in some groups but not others, and this is entirely explained by selection bias, the majority of samples with abnormal thyroid function results having been taken from females. Most importantly ADMA showed no association with homocysteine in any of the groups. Table 5.5 shows the way in which each variable was finally classified as being a predictor of ADMA, SDMA and MMA.

	All samples	Routine homocysteine requests	All samples		Routine homocysteine requests	
	Female and male	Female and male	Female	Male	Female	Male
ADMA						
age	0.067*	0.077*	0.140	0.025**	0.897	0.055*
Na	0.006***	<0.001***	0.020**	0.052*	0.000***	0.005***
total protein	0.002***	0.003***	0.003***	0.166	0.033**	0.019**
fT4	0.001***	0.453	0.001***	0.273	0.637	0.044**
log thcy	0.115	0.299	0.084*	0.255	0.494	0.230
T_r creatinine	0.563	0.461	0.638	0.111	0.202	0.423
T_r uric	<0.001***	<0.001***	0.003***	0.016**	0.002***	0.155
SDMA						
age	<0.001***	<0.001***	<0.001***	0.048**	<0.001***	0.172
Na	0.882	0.096*	0.570	0.938	0.072*	0.696
total protein	0.007***	0.020***	0.152	0.010***	0.456	0.002***
fT4	0.132	0.143	0.536	0.147	0.341	0.062*
log thcy	0.177	0.291	0.627	0.060*	0.849	0.062*
T_r creatinine	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***	<0.001***
T_r uric	0.002***	0.003***	0.688***	0.001***	0.194	0.014**
MMA						
age	0.171	0.389	0.118	0.118	0.291	0.729
Na	0.948	0.856	0.722	0.722	0.516	0.229
total protein	0.046**	0.001***	0.019	0.019	0.001***	0.202
fT4	0.014**	0.114	0.008***	0.008***	0.332	0.455
log thcy	0.382	0.446	0.382	0.382	0.598	0.768
T_r creatinine	0.199	0.007***	0.153	0.153	0.041**	0.106
T_r uric	0.092*	0.615	0.075*	0.075*	0.306	0.594

Table 5.5. Predictors of ADMA, SDMA and MMA as determined by linear modelling. The table gives the significance (probability) of variables being predictors of ADMA, SDMA or MMA. The results for all samples combined and selected subgroups are given for comparison.

* significant at $p < 0.1$
** significant at $p < 0.5$
*** significant at $p < 0.01$

	ADMA	log SDMA	MMA
age	+	+++	–
Na	+++	–	–
total protein	+++	+++	++
T4	+++	–	++
log thcy	–	–	–
Tcreat	–	+++	–
Turic	+++	+++	+

Table 5.6. Classification of variables as predictors of ADMA, SDMA and MMA.

+	=	minor predictor	($p<0.1$)
++	=	moderate predictor	($p<0.5$)
+++	=	significant predictor	($p\leq0.01$)

5.1.5. Discussion of Individual Predictors

5.1.5.1. *Age and Gender*

ADMA, SDMA and MMA show a significant association with age and this, sub-divided by gender, is shown in figure 5.7. ADMA concentrations initially decrease with age, then begin to rise from age 20-30 years before finally reaching a plateau at approximately 70 years of age. In figure 5.7 the data presented is for the results from all samples but the same changes in methylated arginines and age were seen when results from patients with low TSH and high TSH were excluded (not shown). The initial decrease in ADMA concentrations is consistent with the findings of Lücke et al[203] who examined 34 healthy volunteers aged 2 days to 24 years of age and demonstrated that ADMA decreased from approximately 0.8 μmol/L to 0.5 μmol/L over this age range.

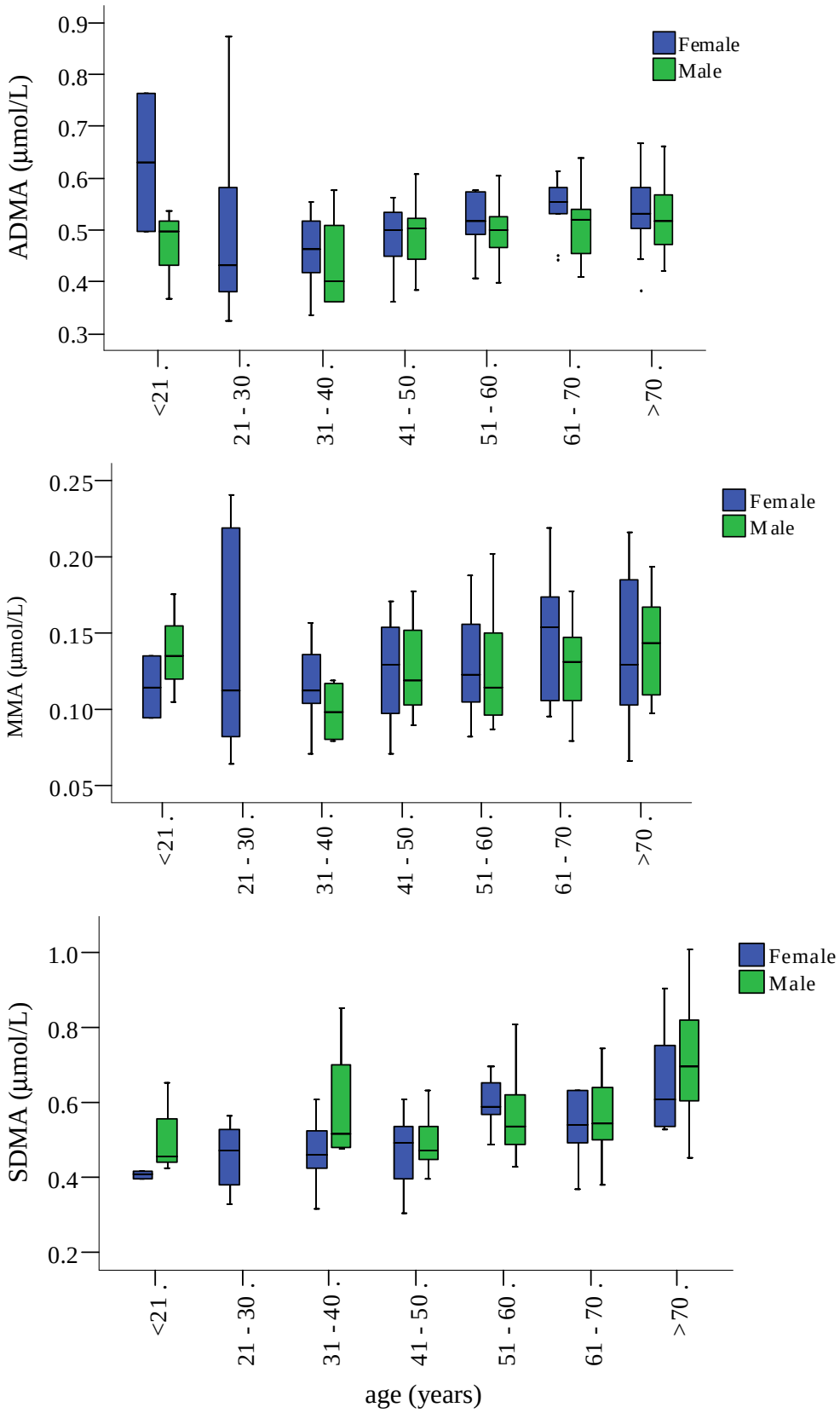


Figure 5.8. Variation of ADMA, SDMA and MMA with age.

In a study of 500 volunteers aged 19 - 75 years Schulze et al also noted an association between plasma ADMA and age[204], however, they concluded that whilst plasma ADMA increases consistently across the age range for men it only shows an increase with age for women post-menopause (greater than 50 years of age). The data from that study is reproduced in figure 5.8 and close inspection would suggest that for women plasma ADMA actually begins to increase before the 50 year age cut-off that had been used, and is in keeping with the data shown in figure 5.7. Schulze et al also concluded that pre-menopausal women had lower plasma ADMA concentrations than men of a similar age, but that following menopause plasma ADMA concentrations in women were higher than men[204]. Figure 5.7 (above) does not substantiate their claim: plasma ADMA concentrations being higher for women compared with men for all age groups. It would seem likely that the single age division used by Schulze et al is inappropriate has led to mis-interpretation of their data.

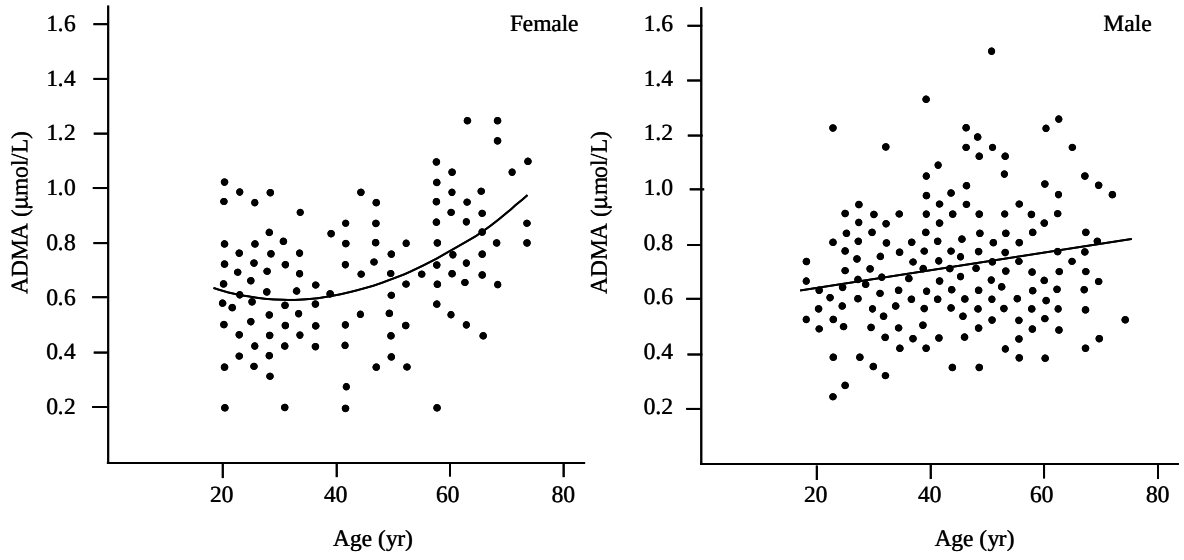


Figure 5.9. Variation of ADMA with age as presented by Schulze et al (2005). Redrawn from reference 204.

MMA possibly shows the same association with age as does ADMA, but it is difficult to draw robust conclusions as there is a higher degree of variability for MMA within each age division compared with ADMA.

SDMA increases with age across the whole age range examined, and it would seem likely that this is a reflection of a decrease in renal function with age, as the renal excretion of SDMA is its only known route of elimination from the body.

5.1.5.2. *Sodium*

Figure 5.9 shows the relationship between plasma ADMA and sodium and although considerable scatter is present (in part due to associations with other variables) the relationship is highly significant (from step-wise regression model $p = 0.006$). An association between ADMA and sodium has been noted previously[205,206,207,208]. In studies on healthy volunteers Kielstein[205,206] et al noted that infusion of ADMA increased urinary retention of sodium, whereas there were no effects on plasma renin or noradrenaline. Plasma sodium was not measured in these studies and it should be noted that, following infusion, plasma ADMA concentrations were greatly increased over normal and pathophysiological values (to approximately 40 μmol/L). In a study of hypertensive subjects, patients followed a low sodium diet for seven days, then a high sodium diet for seven days and finally another low sodium diet for a further seven days. During the periods of low sodium intake plasma concentrations decreased and plasma nitrate increased whilst the reverse was observed during the high sodium diet[207]. The changes in ADMA and nitrate were more pronounced for those subjects deemed to be salt-sensitive (blood pressure increased by >5% by salt loading). In a similar study of normotensive subjects plasma ADMA was again found to increase following sodium-loading and was associated with a decrease in plasma nitrate[208]. When the same subjects followed a high sodium diet but with additional potassium supplementation (60 mmol KCl per day) no changes in either ADMA or nitrate were detected. Although the studies described above confirm the observation of

an association between plasma ADMA and sodium they offer contradictory information as regards to cause and effect. In the present work the strength of association seen between sodium and ADMA is somewhat surprising given that the samples came from an essentially random selection of patients who were not deliberately stressed with either salt or ADMA loading. The effect of sodium on blood pressure in the general population remains debatable[209], but patients with end-stage renal failure are particular susceptible to the adverse effects of salt loading which can lead to severe hypertension and left ventricular hypertrophy[209]. It has been suggested that these adverse effects due to the increased plasma sodium itself and not due to the associated hypervolaemia[209] and, given this, further research into the link between plasma ADMA and sodium is warranted.

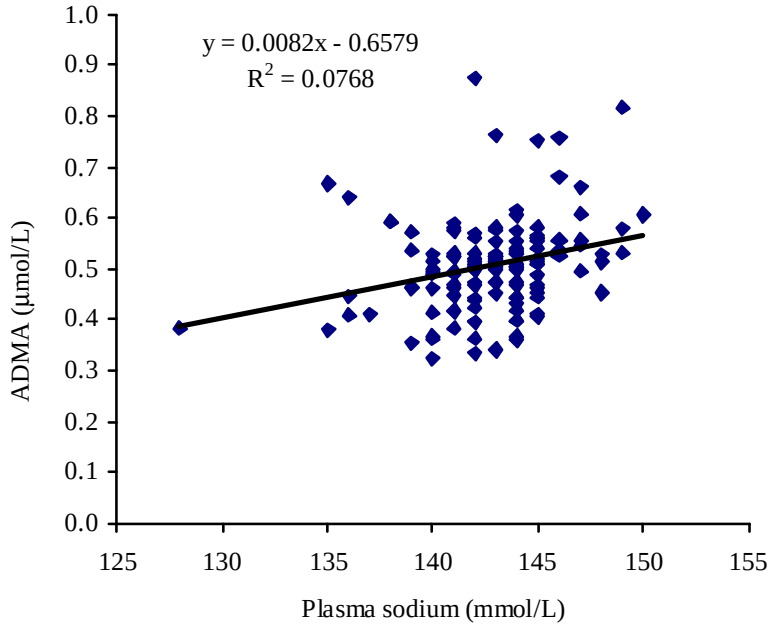


Figure 5.10. The variation of plasma ADMA with plasma sodium. The Pearson correlation coefficient (r = 0.277) is highly significant at p = 0.01.

5.1.5.3. *Thyroid Function*

The associations between ADMA, MMA, log SDMA and TSH and fT4 are shown in figure 5.11. ADMA was significantly increased for patients with decreased TSH as compared with patients with TSH values within the reference range (Mann-Whitney, $p = 0.001$), no difference was detected for patients with an increased TSH compared with those with normal TSH (Mann-Whitney, $p = 0.904$). ADMA concentrations showed a significant association with fT4 ($p = 0.004$). Similar results were observed for MMA, being increased for decreased TSH as compared to normals (Mann-Whitney, $p = 0.001$), no difference between patients with high TSH as compared with normals (Mann-Whitney, $p = 0.816$) and a significant association with fT4 ($p = 0.024$). No statistically significant differences were detected for log SDMA for low vs normal TSH (Mann-Whitney, $p = 0.142$) or high vs normal TSH (Mann-Whitney, $p = 0.730$), however a statistically significant association was present for log SDMA and fT4 ($p = 0.004$); this latter finding was inconsistent with the results obtained with linear modelling in which no association was detected between log SDMA and fT4. Further analysis of the data showed a significant association between creatinine and fT4 ($T_r$creatinine vs fT4, Pearson correlation = 0.027), and when linear modelling for log SDMA was repeated, with the exclusion of creatinine as a variable, fT4 became a significant predictor of log SDMA ($p = 0.019$). A possible explanation of these findings is that there are changes in GFR, associated with thyroid status, with an associated change in the renal excretion of SDMA, and that SDMA concentrations are therefore indirectly linked with thyroid status. To further investigate this possibility results from routine requests for creatinine and thyroid function for a one month period were extracted from the laboratory computer system. The creatinine results were divided into seven groups according to TSH, with five groups being used to cover the TSH reference range (0.3 - 4.2 mu/L); results were only used for patients with a normal eGFR (>60 ml/min) and males and females were analysed separately. The results of the analysis are shown in table 5.6 and the confidence intervals for the mean creatinine of each group in figure 5.11. Absolute differences between the groups are small but statistically highly significant (one-way ANOVA, females: $p < 0.001$, males: $p = 0.001$). When log SDMA was grouped in a similar fashion according to TSH (figure 5.12) one-way ANOVA showed a statistically significant association between the two ($p = 0.031$).

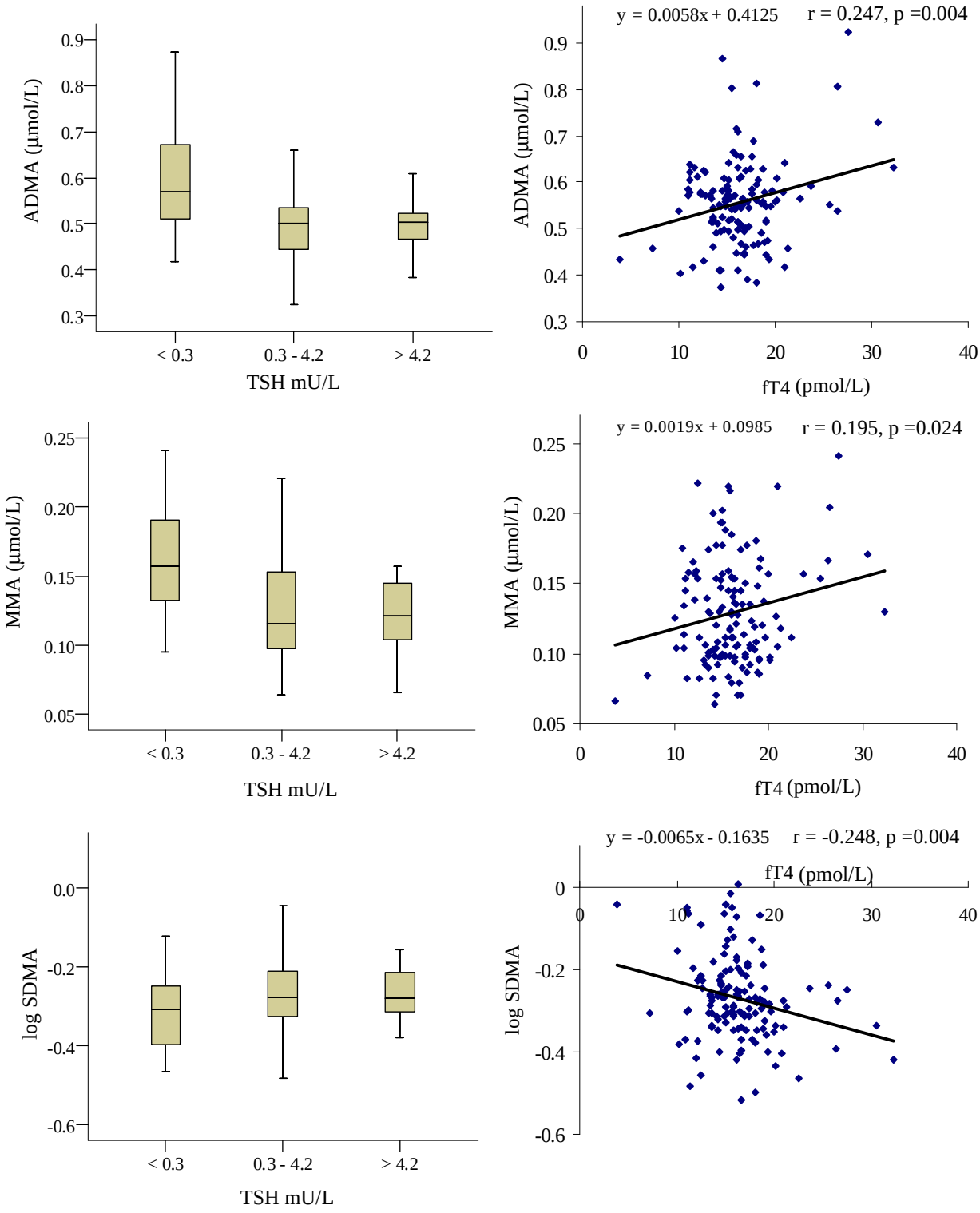


Figure 5.11. The variation of plasma ADMA, MMA and SDMA with TSH and with fT4. For patients with low vs normal TSH a statistically significant increase for both ADMA and MMA was observed (Mann-Whitney: p = 0.001 for both). No significant difference was detected for patients with a high vs normal TSH for either ADMA or MMA (Mann-Whitney: p = 0.904 and p = 0.816 respectively). For log SDMA no significant difference was observed for either patients with low vs normal TSH or high vs normal TSH (Mann:Whitney: p = 0.142 and p = 0.730 respectively). ADMA, MMA and log SDMA were all highly correlated with fT4 (Pearson's rho, p =0.247, p = 0.024 and p = 0.004 respectively).

TSH (mU/L)	Females n	Females mean plasma creatinine (μmol/L)	Males n	Males mean plasma creatinine (μmol/L)
<0.30	121	64.5	45	78.7
0.30 - 1.08	413	64.1	280	83.0
1.09 - 1.86	728	66.0	656	84.3
1.87 - 2.64	587	67.1	545	85.3
2.65 - 3.42	344	67.4	274	84.9
3.43 - 4.20	192	68.6	160	85.7
> 4.20	353	68.2	193	86.6
total	2738		2153	

Table 5.7. The variation of plasma creatinine with TSH. The results of routine requests for plasma creatinine and TSH for a one month period were extracted from the laboratory computer system and grouped according to TSH such that the TSH reference range (0.3 - 4.2 mU/L) has been divided into five equally spaced groups.

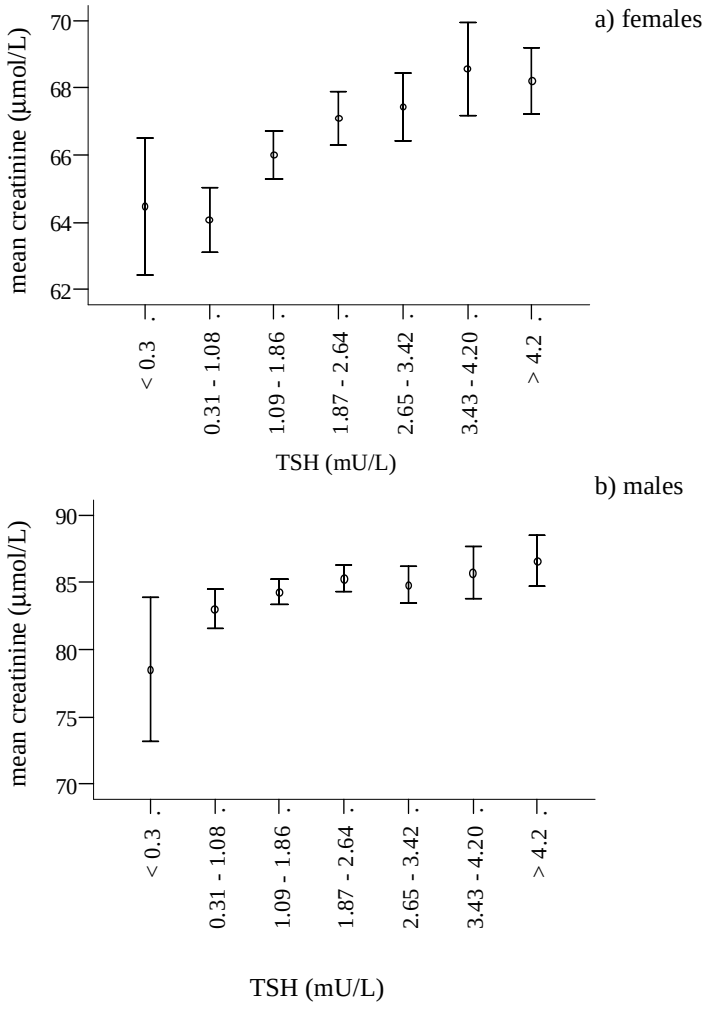


Figure 5.12. The confidence interval of the mean for plasma creatinine. The results were grouped by TSH, such that the TSH reference range (0.3 - 4.2 mU/L) has been divided into five equally spaced groups. One way ANOVA showed a highly significant variation of mean plasma creatinine with TSH, for both females ($p < 0.001$) and males ($p = 0.01$) using data for routine requests extracted from the laboratory computer system for a one month period.

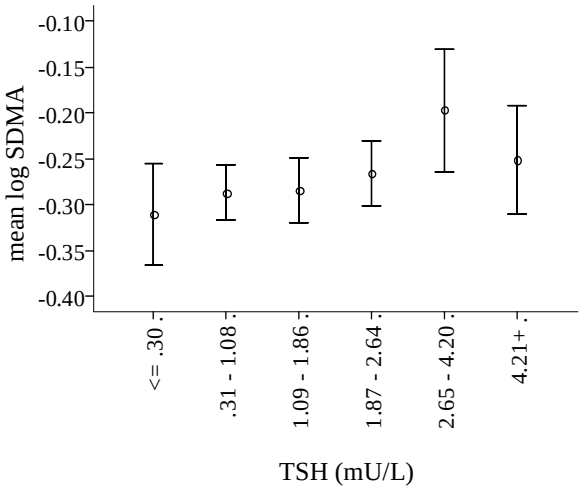


Figure 5.13. Confidence of the mean for log SDMA. The results have been grouped according to TSH such that the TSH reference range (0.3 - 4.2 mU/L) has been divided into five equally spaced groups. One way ANOVA showed a significant variation of mean log SDMA with TSH ($p = 0.031$).

Two studies on the association between ADMA, SDMA and thyroid function been published[210,211] and the findings, with comparison with the present work, are summarised in table 5.7. ADMA is consistently shown to be increased in hyperthyroidism, however a difference in ADMA between hypothyroid and euthyroid patients (increased ADMA in hypothyroidism) was only observed by Arikan et al. There is a complete lack of consistency regarding observations between SDMA and thyroid status, however, as noted above, associations between SDMA and TSH appear to depend greatly upon the way in which results have been subdivided; Hermenegildo et al having used TSH concentrations > 15 mU/L to classify patients as hypothyroid and Ariken et al using a threshold of 10 mU/L. It should also be noted that in the present work, patients in the hyperthyroid group did not have primary hyperthyroidism, unlike the patients included by Hermenegildo et al and Ariken et al, but instead had a hyperthyroid pattern of results due to being over-treated hypothyroid patients.

The reason for the association between ADMA and thyroid status has not been determined but a possible explanation comes from cell culture studies in which it has been

demonstrated that the activity of PRMT I (and therefore production of ADMA) is regulated by T3 concentration[212].

Status	ADMA			SDMA		
	current work	Hermenegildo et al[210]	Arikan et al[211]	current work	Hermenegildo et al[210]	Arikan et al[211]
hyperthyroid	0.60 ± 0.12 ↑ [a)] (n = 16)	1.30 ± 0.12 ↑ [a)] (n = 19)	0.89 ± 0.28 ↑ [a)] (n = 25)	0.50 ± 0.12 (n = 16)	0.54 ± 0.07 ↑ [a)] (n = 19)	0.35 ± 0.11 (n = 25)
euthyroid	0.49 ± 0.08 (n =104)	0.58 ± 0.06 (n = 31)	0.40 ± 0.08 (n = 25)	0.56 ± 0.14 (n = 104)	0.27 ± 0.04 (n = 31)	0.41 ± 0.11 (n = 25)
hypothyroid	0.49 ± 0.07 (n = 14)	0.57 ± 0.07 (n = 12)	0.69 ± 0.45 ↑ [a)] (n = 23)	0.58 ± 0.15 (n = 14)	not given	0.28 ± 0.11 ↑ [a)] (n = 23)

Table 5.8. Comparison of findings regarding thyroid status and methylated arginines with published studies. ADMA is consistently shown to be increased in hyperthyroidism, however there are inconsistencies as to findings in patients with hypothyroidism. There is no consistency with regard to the findings for SDMA, however it should be noted that in the current work, when SDMA is further subdivided according to TSH, it shows a significant increase with TSH.

[a)] ↑ significant increase ($p \leq 0.01$)

5.1.5.4. *Uric Acid*

The associations between methylated arginines and transformed uric acid are shown in figure 5.13. ADMA and log SDMA both showed highly significant associations with transformed uric acid when both correlation coefficients were examined ($p = 0.001$ and $p < 0.001$ respectively) and linear modelling was used ($p < 0.001$ and $p = 0.002$ respectively. An association between MMA and transformed uric acid was only noted when linear modelling was used and was only of slight significance ($p = 0.092$). The association between ADMA, SDMA and uric acid is most probably due to the fact that they share a similar origin; ADMA and SDMA resulting from the breakdown of nuclear proteins and the bulk of uric acid being formed from the metabolism of endogenous nucleic acids[213].

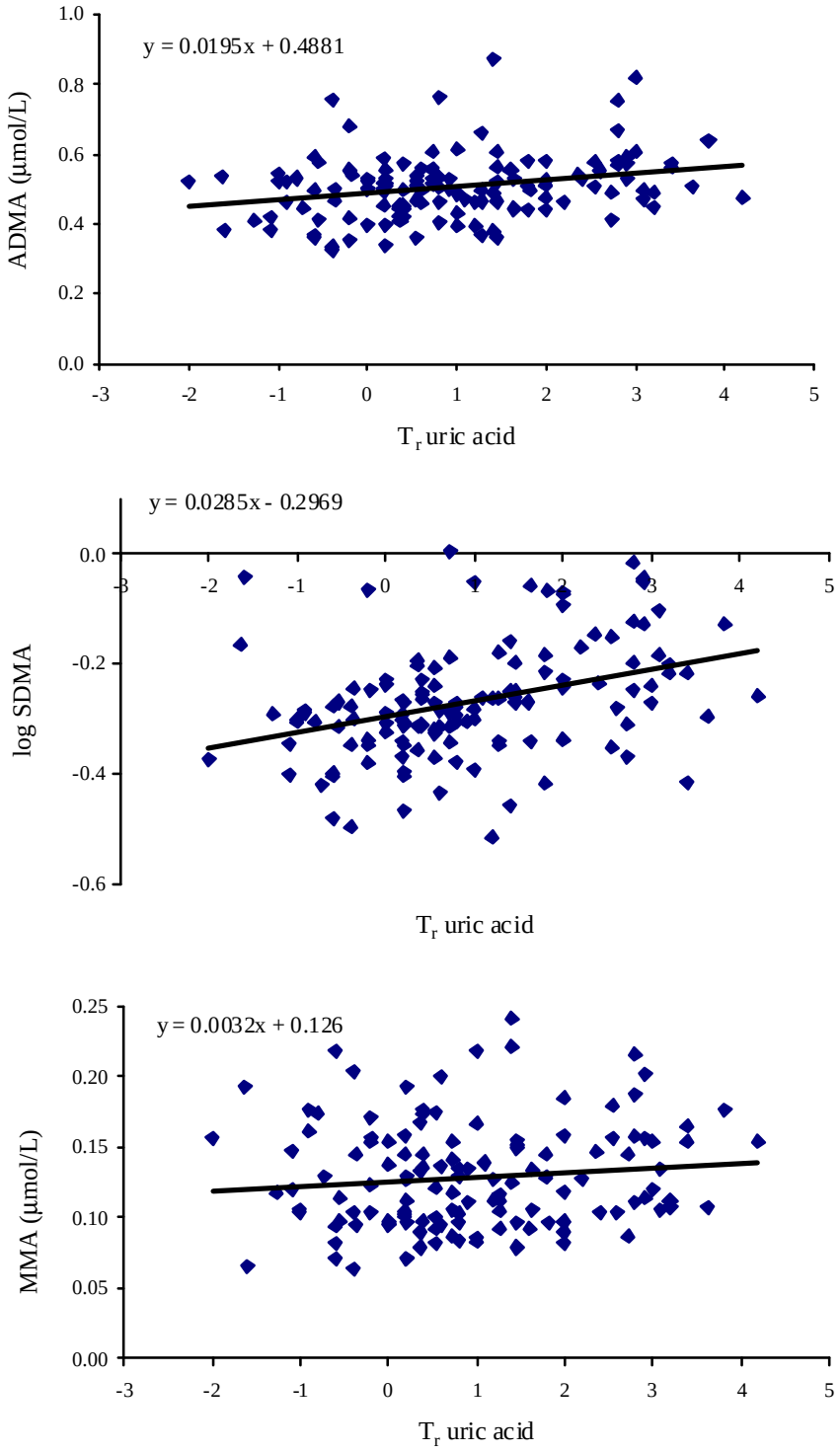


Figure 5.14. The association between uric acid and methylated arginines. The methylated arginines are plotted against transformed uric acid. ADMA and log SDMA both showed statistically significant correlations with transformed uric acid (Pearson: p = 0.001 and p 0.001 respectively. No significant association was detected between MMA and transformed uric acid (p = 0.200).

5.2. The ACADEMIC Study

The ACADEMIC (arterial compliance and oxidant stress as predictors of rate of decline of renal function, morbidity and mortality in chronic kidney disease) study is an ongoing prospective investigation of cardiovascular risk in CKD stages 3 and 4 designed to evaluate the relationship between changes in arterial stiffness and renal function over time. The clinical aspects of this study were conducted by Dr Laurie Tomlinson (LT; Research Fellow, Brighton & Sussex University Hospitals NHS Trust).

5.2.1. Subjects and Investigations

Subjects

All participants enrolled in the study were classified as having CKD stages 3 or 4 (staged according to the 2005 UK CKD guidelines). Exclusion criteria were previous diagnosis of left ventricular failure, aortic stenosis with gradient >30 mmHg and uncontrolled atrial fibrillation. All participants were treated with the aim of achieving United Kingdom Renal Association targets for management of BP in CKD; a target BP of 130/80 mmHg or less for patients with a urine protein:creatinine ratio <100 mg/mmol and 125/75 mmHg for those with a protein:creatinine ratio >100 mg/mmol. The choice of antihypertensive medication was at the discretion of the patient's clinician but followed Renal Association and British Hypertension Society guidelines. The study was approved by the West Sussex Research Ethics Committee, and the patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

Patients who gave written informed consent for participation were included and had baseline clinical assessment, laboratory and PWV measurements and follow-up at 6 and 12 months. A full history of renal disease, cardiovascular disease and associated risk factors was obtained. All measurements were conducted in a quiet, temperature controlled room by the same two researchers throughout the study. Cardiovascular disease was defined as a history of myocardial infarction, angina, coronary artery bypass grafting, stroke, transient ischaemic attack or peripheral vascular disease given by the patient and confirmed from the

medical notes. The cause of renal disease was determined by review of the medical notes, including biopsy results, imaging and blood tests, by the renal medical team. Hypertension was defined as a blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic and/or the current use of antihypertensive medication.

PWV recordings

Aortic stiffness was assessed by automatic carotid-femoral PWV measurement using the Complior device (Artech-Medical, Paris, France); the technical characteristics of this device have been described previously[214], and indicate intra-observer within-session coefficients of variation for aortic PWV of 3.8 ±1.3% (*n*=25) In-house assessment of analytical reproducibility suggested an intra-observer imprecision of less than 4%.

"Briefly, common carotid artery and femoral artery pressure waveforms were recorded noninvasively with a pressure-sensitive transducer. The pressure waveforms were digitised at a sample acquisition frequency of 800 Hz. The two pressure waveforms were then stored in a memory bank. A preprocessing system automatically analysed the gain in each waveform and adjusted it to equalise the two signals. Details of this procedure have been published previously (Asmar *et al.*, 1995[214]). When the operator observed a pulse waveform of sufficient quality on the computer screen, digitisation was suspended and calculation of the time delay between the two pressure upstrokes was initiated. Measurement was repeated over 10 different cardiac cycles, and the mean was used for the final analysis. The distance travelled by the pulse wave was measured over the body surface as the distance between the two recording sites (D), whereas pulse transit time (t) was determined by the Complior. PWV was automatically calculated as PWV = D/t."
(Provided by Dr Laurie Tomlinson)

Blood pressure measurements

Oscillometric BP was measured twice on the right arm using an appropriate cuff size with the patient supine after 5 and 10 min of rest (Omron 705 CP, Tokyo, Japan), and the mean of the two recordings of SBP and DBP was recorded. The mean blood pressure (MBP) was calculated by MBP = (SBP − DBP)/3 + DBP.

Biochemical analysis

At baseline, fasting lithium heparinised plasma samples were taken to measure creatinine, albumin, total cholesterol and uric acid. Plain random urine samples were also obtained and used to estimate proteinuria (total protein:creatinine ratio where > 30 mg/mmol was considered significant).

Plasma cystatin-C concentrations were measured using a BNProSpec nephelometer (Dade Behring, Inc., UK) with a particle-enhanced immunonephelometric assay (N Latex Cystatin-C, Dade Behring, Inc.)

Estimated glomerular filtration rate (eGFR), ml/min/1.73m^2 body surface area, was calculated using the four-variable modified MDRD formula:

$$\text{eGFR} = 186 \times ([\text{Creatinine}]\ (\mu\text{mol/l})) / 88.4)^{-1.154} \times (\text{Age (years)})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$$

Study Population

Baseline characteristics are summarised in table 5.8. None of the participants were receiving dialysis at baseline. The etiology of kidney disease was due to polycystic kidney disease in 23%, glomerular disease in 38%, and other causes in 39%.

Parameter	Mean ± sd *or* median (IQR)	Reference range (at Royal Sussex County Hospital, Brighton)
Demographics		
age (years)	69.1±11.5	
Male (%)	77	
Medical History		
History of vascular disease (%)	42.9	
History of hypertension (%)	78	
History of diabetes (%)	23	
History of smoking (%)	65	
Body mass index (kg/m^2)	29.4 ± 5.8	
Clinical Parameters		
Systolic Blood Pressure (mmHg)	154.7 ± 20.9	
Diastolic BP (mmHg)	82.7 ± 11.4	
Pulse Pressure (mmHg)	71.8 ± 19.6	
MAP (mmHg)	106.6 ±12.3	
CF-PWV (m/s)	12.6 ± 2.8	
Plasma biochemistry		
eGFR (mL/min/1.73m^2)	32.1 ± 10.9	≥ 60
creatinine (F) (μmol/L)	169.4 ± 56.3	44 - 80
creatinine (M) (μmol/L)	210.8 ± 69.5	62 - 106
Transformed creatinine	11.62 ±5.96	
cystatin C (mg/L)	1.86 ± 0.58	0.49 - 0.98
albumin (g/L)	42.5 ± 3.1	34 - 48
cholesterol (mmol/L)	4.37 ± 0.98	< 5.0
uric acid (F) (mmol/L)	0.423 ± 0.118	0.14 - 0.34
uric acid (M) (mmol/L)	0.468 ± 0.096	0.20 - 0.42
Transformed uric acid	2.98 ± 1.92	
ADMA (μmol/L)	0.554 ± 0.102	
SDMA (μmol/L)	0.997 (0.821 - 1.310)	
MMA (μmol/L)	0.171 (0.147 - 0.204)	
arginine (μmol/L)	88 (72 - 121)	
total homocysteine (μmol/L)	22.2 (18.8 - 27.3)	5 - 15
Urine biochemistry		
protein:creatinine ratio (mg/mmol)	28 (14 - 70)	
Medication use		
anti-hypertensives (%)	90	
diuretics (%)	60	
statins (%)	60	

Table 5.9. Baseline characteristics of 133 patients in the ACADEMIC study.

5.2.2. Results

SDMA, MMA, arginine and total homocysteine were not normally distributed (Kolmogorov-Smirnov: $p = 0.044$, 0.009, 0.021 and 0.47 respectively) but following logarithmic transform each variable passed the test for normality (Kolmogorov-Smirnov: $p = 0.754$, 0.791, 0.564 and 0.831 respectively).

No association was found between any biochemical parameter and age, history of smoking, BMI, CF-PWV or any of the various measures of blood pressure.

Biochemical parameters which showed an association with ADMA, log SDMA, log MMA and/or log total homocysteine are shown in table 5.9, no other significant associations ($p \leq 0.05$) were noted.

Log total homocysteine and log SDMA both showed strong associations with all measures of renal function ($T_r$creat, eGFR and cystatin C), consistent with previous findings[53,117]. ADMA, however, showed a very significant association with cystatin C, but not with $T_r$creat or eGFR (significance of Pearson correlation coefficient: $p < 0.001$, $p = 0.247$, $p = 0.403$, respectively). The association between ADMA and cystatin C is most probably due to the fact that both are derived from the degradation of nucleic proteins[103,215]. No statistically significant association was found between ADMA and log homocysteine.

To investigate which biochemical parameters might be determinants of CF-PWV a multivariate stepwise model was constructed using age, ADMA, log SDMA, log arginine, log MMA, log (arginine/ADMA), log total homocysteine, $T_r$uric acid, $T_r$creat, and cystatin C as variables. Log (arginine/ADMA) was included as it has been suggested that the ratio of arginine to ADMA is a determinant of vascular function[216]; the ratio of arginine to ADMA did not passed the Kolmogorov-Smirnov test for normality ($p = 0.006$) whereas the logarithm of the ratio did ($p = 0.719$).

The only significant determinants of CF-PWV were age and cystatin C ($p < 0.001$, $p = 0.04$ respectively).

	Pearson correlation analysis	log hcy	ADMA	log SDMA	log MMA	log arginine
log thcy	Correlation Coefficient		0.153	0.253	0.061	0.118
	Significance		0.078	0.003**	0.484	0.177
ADMA	Correlation Coefficient	0.153		0.355	0.482	0.146
	Significance	0.078		<0.001**	<0.001**	0.094
log SDMA	Correlation Coefficient	0.253	0.355		0.210	0.169
	Significance	0.003**	<0.001**		0.015*	0.052
log MMA	Correlation Coefficient	0.061	0.482	0.210		0.320
	Significance	0.484	<0.001**	0.015*		<0.001**
log arginine	Correlation Coefficient	0.118	0.146	0.169	0.320	
	Significance	0.177	0.094	0.052	<0.001**	
albumin	Correlation Coefficient	0.155	-0.182	-0.162	-0.067	-0.037
	Significance	0.075	0.036*	0.062	0.441	0.674
eGFR	Correlation Coefficient	-0.344	-0.073	-0.716	-0.093	-0.060
	Significance	<0.001**	0.403	<0.001**	0.284	0.495
Transformed creatinine	Correlation Coefficient	0.288	0.101	0.746	0.082	0.136
	Significance	<0.001**	0.247	<0.001**	0.348	0.119
cystatin C	Correlation Coefficient	0.319	0.309	0.727	0.071	0.104
	Significance	<0.001**	<0.001**	<0.001**	0.415	0.233
Transformed uric acid	Correlation Coefficient	0.236	-0.019	0.152	0.047	0.010
	Significance	0.006**	0.829	0.081	0.594	0.913

Table 5.10. Correlations between plasma results for participants in the ACADEMIC study.

** significant at $p < 0.5$
*** significant at $p < 0.01$

5.2.3. Discussion

Plasma ADMA showed no association with log total homocysteine for either samples that had been received for routine clinical investigation or for samples that had been taken as part of the ACADEMIC study. For the samples received routinely, effort had been made to ensure that plasma had been promptly separated from red-cells, however possible delays in the receipt of these samples meant that this could not be guaranteed. Erythrocytes continue to produce homocysteine after blood samples have been taken[53], and delays in sample processing could be a confounding factor in the analysis, however this would not apply to the results from the ACADEMIC study, in which samples had been processed immediately. If plasma total homocysteine concentrations are a determinant of plasma ADMA concentrations then, the fact that no association has been found between the two in this work, it would appear that it is a much weaker determinant than other factors such as plasma sodium concentrations and thyroid status.

For patients in the ACADEMIC study no association was found between CF-PWV and total homocysteine concentrations; results from other studies (section 1.4.3) have reported both negative and positive associations. A large percentage of patients in the ACADEMIC study were taking statins (60%), diuretics (60%) and/or anti-hypertensive agents (90%) and the use of these medications may have been a confounding factor in the investigation. There was not sufficient data for patients taking no medication to perform a sub-group analysis.

In conclusion, the results of this work do not support the hypothesis that elevated plasma homocysteine concentrations cause vascular dysfunction by increasing plasma ADMA.

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Simultaneous quantitation of homocysteine, cysteine and methionine in plasma and urine by liquid chromatography-tandem mass spectrometry. G Weaving, BF Rocks, SA Iversen, MA Titheradge. Ann Clin Biochem (2006) 43, 474-480.

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Arginine and methylated arginines in human plasma and urine measured by tandem mass spectrometry without the need for chromatography or sample derivatisation. G Weaving, BF Rocks, MP Bailey, MA Titheradge. J Chrom B (2008) 874. 27-32.

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